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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

We investigated the role of nitric oxide (NO) in MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-induced dopaminergic (DA) neuron death in this mouse model of Parkinson's Disease (PD). Our previous work demonstrated that the superoxide radical is involved in the MPTP neurotoxic process in SNpc DA neurons. SNpc DA neurons in mice overexpressing superoxide dismutase (SOD1) were protected against MPTP-induced degeneration. Since the superoxide radical does not act alone, based on the oxidative stress hypothesis, in **Specific Aim I**, we demonstrated that neuronal NOS (nNOS) and inducible NOS (iNOS) are both players in MPTP-induced neurotoxicity to SNpc DA neurons. In **Specific Aim II**, we show that while nNOS has a role in MPTP toxicity to SNpc DA neurons, iNOS is the principle culprit here due to its upregulation in activated microglia. Peroxynitrite-induced alterations in protein tyrosine residues were demonstrated in striatum and ventral mibrain of MPTP-treated mice by measurement of nitrotyrosine, orthotyrosine and o,o-dityrosine in **Specific Aim III**. Furthermore, **Specific Aim IV** showed that proteins important to normal function in SNpc DA neurons, tyrosine hydroxylase and α-synuclein, are nitrated following MPTP exposure. We conclude that the superoxide radical and NO act in concert to initiate and propagate DA neuronal death in the SNpc of MPTP-treated mice and in PD patients.

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INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disorder that affects about 1,000,000 North Americans alone and that occurs in 50,000 newly diagnosed patients each year (1). This disease is characterized mainly by tremor, rigidity, akinesia and postural instability (2) all attributed to a dramatic loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and of DA nerve terminals in the caudate-putamen (3,4). Symptoms of PD can be alleviated with the use of levodopa and other dopamine agonists, however, they do not stop the progression of the disease. Many hypotheses abound as to the etiology of PD, however, it is the oxidative stress theory that seems to take precedence over the others. In previous studies (5,6), we have shown that reactive oxygen species (ROS) and reactive nitrogen species are involved in the PD neurodegenerative process. To demonstrate the involvement of these compounds in the PD neurodegenerative process, we have used 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a by-product of the chemical synthesis of a meperidine analogue with potent heroin-like effects (7). MPTP is a specific DA neurotoxin that replicates almost all of the hallmarks of PD in non-human primates and in various other mammalian species including a severe irreversible loss of DA neurons in the SNpc and a loss of DA terminals in the caudate nucleus (8). Using transgenic mice that overexpress human superoxide dismutase (SOD), the enzyme responsible for metabolizing the superoxide radical in the cell, we have demonstrated that the superoxide radical participates in the MPTP neurotoxic process (5). However, superoxide is poorly reactive and it is the general consensus that this radical by itself does not cause serious damage. It is believed that superoxide exerts most of its toxic effects through the generation of other reactive species such as the hydroxyl radical whose oxidative properties can ultimately kill cells. For instance, superoxide facilitates hydroxyl radical production through its reaction with hydrogen peroxide and transition metals. Superoxide radical can also react with nitric oxide (NO) to produce the extremely potent and more damaging peroxynitrite Production of NO is the result of activation and upregulation of the NO-producing enzyme, nitric oxide synthase (NOS). Three distinct isoforms of nitric oxide synthase (NOS) that synthesize NO in the brain (7), neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) are either constitutively expressed (nNOS, 7) or minimally expressed (eNOS, iNOS, 7). Working with this aspect of the oxidative stress hypothesis, we and others (5,9,10) have found that knocking out nNOS only partially protects against MPTP's toxic effects. From this, we surmised that other NOS isoforms might indeed play some role in the MPTP neurotoxic effect and in PD, thus the entry of iNOS. In the normal brain, iNOS is undetectable or only minimally expressed. However, in pathological conditions such as stroke, AIDS, ALS and PD (11-14), it has been shown that only iNOS is upregulated. In fact, it has been suggested that the iNOS expression that has been demonstrated in microglia in the SNpc of post-mortem brain tissues from PD patients indicates that an inflammatory process may influence the nature of PD. It is postulated that while iNOS is not instrumental in the initiation of PD, it may well aid in the progressive nature of the disease. In another experiment using several immunostaining techniques and western blot analyses, we investigated in mouse brain the

role of iNOS in the MPTP neurotoxic process. Our findings that iNOS is up-regulated in the SNpc of MPTP-treated mice (15) is more relevant to our finding that the superoxide radical is involved in the death of DA neurons following MPTP treatment than our results with nNOS gives teeth to our hypothesis that other NOS isoforms are involved here. Once we demonstrated that iNOS upregulation is tied to microglial activation, we thought that other pro-inflammatory agents which are involved in the inflammatory response (16) might be involved here also. Furthermore, we thought that the blockade of iNOS upregulation might be a possible therapeutic avenue for attenuation of the MPTP neurotoxic process. Thus, we used minocycline, a semisynthetic tetracycline derivative to attenuate microglial activation (17). As stated above, the superoxide radical and NO are weak oxidants, and individually, neither is sufficiently damaging. However, reaction between the two can produce the more damaging peroxynitrite (18), a reactive nitrogen species that can oxidixe the phenolic rings, particularly tyrosine, of proteins and damage DNA (19,20). Markers for the oxidation of tyrosine, nitrotyrosine and dityrosine, are indicative of the extent of the peroxynitrite-induced damage to tyrosine residues which are so important to phosphorylation, signal transduction and catecholamine synthesis in the cell (21).

As mentioned above, there are three isoforms of the NOS enzyme. In models of focal ischemia, nNOS mediates early neuronal injury and iNOS contributes to late neuronal injury, but eNOS is thought to be neuroprotective (18). The production of NO from eNOS supposedly protects brain tissue by maintaining regional cerebral blood flow(18). Thus, to complete our studies on the contribution of NOS isoforms to SNpc MPTP neurotoxicity, we examined the effects of MPTP on the eNOS enzyme in wild-type and eNOS knockout mice and found that eNOS has no role in the MPTP-induced neurotoxic process (Personal Communication).

The next step in our research plan is to demonstrate whether we can alter the MPTP-induced toxic process on SNpc DA neurons by altering superoxide radical and/or NO production as we believe that these alterations may dampen this neurotoxic effect on SNpc DA neurons. To this end, we theorize that it is necessary to alter superoxide radical and/or NO production in the face of MPTP administration. For this, we felt that overexpressing SOD and/or knocking out NO production via transgenic and knockout mice might do the trick. We have found that cross breeding SOD overexpressers with nNOS and iNOS knockout mice is feasible and these mice are viable. Exploring the effects of MPTP in these mice will help us further our theories of oxidative stress and PD.

BODY OF RESEARCH

Our overall long-term goal is the study of the pathogenesis of PD based on the oxidative stress hypothesis of PD. Thus, we have outlined a series of studies using the specific DA neurotoxin MPTP that hopefully will help us to decipher, in a step by step fashion, the cascade of events that cause this debilitating disease and, along the way, possible therapies to improve the symptomology of or to stop the progression of PD. Our research plan has hooked into strong support for the oxidative stress hypothesis of PD. Data from our MPTP studies have not only replicated a number of hallmarks so characteristic of PD

such as the severe loss of DA cell bodies in the SNpc of MPTP-treated mice along with a marked decrease in DA fibres in the striatum but have also demonstrated that both the superoxide radical and NO are involved in the production of the strong oxidant peroxynitrite which can damage proteins and DNA **(Specific Aim I)**. For instance, we have demonstrated the oxidative modification and nitration of tyrosine residues and alpha-synuclein using our MPTP mouse model of PD **(Specific Aim IV)**. These nitrated compounds have been demonstrated in post-mortem tissues from PD brains. Our studies also show that eNOS is not at all involved in the MPTP neurotoxic process and that iNOS activation and upregulation are key to the MPTP neurotoxic process **(Specific Aim II)** in that the above-mentioned proteins are nitrated which takes them out of the loop of normal cell function. We have also shown that the main products of peroxynitrite oxidation of tyrosine residues in our MPTP mouse are the formation of dityrosine, o,o-dityrosine and 3-nitrotyrosine **(Specific Aim III)**. Our research now directs us toward attempts to dampen the effects of superoxide radical and NO production following MPTP administration using transgenic mice that overexpress SOD and knockout mice that lack the nNOS and the iNOS enzymes.

Animals and treatment

Procedures using laboratory animals are all in accordance with the NIH guidelines for use of live animals and are approved by the institutional animal care and use committee of Columbia University. Eight-week-old female C57BL/6 female mice (Charles River Laboratories, Wilmington, Massachusetts) are being bred with our SOD overexpressers to put the SOD mice on a C57BL/6 background. nNOS and iNOS knockout mice are on a C57BL/6 background from Jackson Laboratories. The two NOS knockout lines were crossed with the SOD mice for viability. These mice are viable, thus, we will breed them for the remaining MPTP experiments. SOD gel electrophoresis and RT-PCR are used to establish genotype.

For all of our previous experiments using MPTP, we have used 8-10 weeks old C57BL/6 male mice from Charles River Laboratories. These mice received a 18-20mg/kg injection of MPTP at two hour intervals (4 doses over 8 hours) and their brains were used to test our hypothesis. The nigrostriatal DA system of C57BL/6 mice is highly susceptible to the toxic effects of MPTP as evidenced by the 80-90% reduction in mouse striatal DA content, by the 60-70% loss of DA neurons in the substantia nigra pars compacta (SNpc) and by severe damage to DA terminals in the striatum which is the same situation that exists in human PD.

Overall Accomplishments for Specific Aim I.

- iNOS and nNOS are both involved in MPTP-induced toxicity to DA neurons.
- iNOS seems to be the culprit in the activation of microglia and the progression of MPTP-induced DA neurodegeneration.
- eNOS is not involved in the MPTP-induced neurotoxic process.

- Crosses between the SOD overexpressers and nNOS and iNOS knockout mice are viable.

Overall Accomplishments for Specific Aim II

We have shown that minocycline, a semisynthetic tetracycline derivative, attenuates certain aspects of the MPTP-induced inflammatory response in mice. These include:

- Attenuation of MPTP-induced SNpc dopaminergic neuron death by minocycline.
- Prevention of MPTP-induced microglial activation by the second generation semisynthetic antibiotic minocycline.
- Prevention of three key microglial-derived mediators of cytotoxicity following MPTP administration using minocycline. These include iNOS upregulation, formation of mature IL-1β and activation of NADPH oxidase.

Overall Accomplishments of Specific Aim III.

- Documentation of peroxynitrite existence and regional quantification of 3-nitrotyrosine, ortho-tyrosine and o,o-dityrosine in the MPTP mouse model of Parkinson's Disease.
- Demonstration that tyrosine hydroxylase is nitrated and inactivated following MPTP administration to mice.

Overall Accomplishments of Specific Aim IV.

- Demonstration of tyrosine hydroxylase inactivation and tyrosine nitration in HEK293 cells following exposure to peroxynitrite.
- Demonstration of the nitration and oxidative modification of the α-synuclein protein in the SN of MPTP-treated mice.
- Demonstration that poly (ADP-ribose) polymerase, a DNA repair enzyme, is upregulated in the ventral midbrain of MPTP-treated mice.

Reportable Outcomes

Specific Aim I

Specific Aim I of this award proposed to determine the contributions of superoxide, NO or both to MPTP neurotoxicity by administering this toxin to normal mice and to different lines of mice that are genetically engineered to exhibit a greater capacity for detoxifying superoxide (transgenic copper/zinc-superoxide dismutase {SOD1} mice) and/or a lower capacity for synthesizing NO (knockout neuronal NO synthase {nNOS} mice and by assessing the status of the nigrostriatal DA pathway in these different types of mice following MPTP administration using high performance liquid chromatography (HPLC) and immunostaining with quantitative morphology. We have already completed our studies on the contribution of the three isoforms of the NOS enzyme to the MPTP neurotoxic process and have found that both nNOS and iNOS contribute to MPTP-induced toxicity on dopamine neurons in accordance with **Specific Aim I.** We also note that eNOS plays no role in this MPTP-induced toxicity. In keeping with **Specific Aim I**, this year, thus far, we have put the mice necessary for other aspects of **Specific Aim I** on a C57BL/6 mouse background as we have used the C57BL/6 mouse for our original MPTP studies. We have also started to cross the SOD overexpressers with nNOS and iNOS knockout mice in order to complete the remainder of **Specific Aim I.** We have found that the SOD/NOS knockout mice are viable. 5-6 animals per group are needed for each study which requires breeding a large number of crossed animals.

Publications Relevant to Specific Aim I

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Specific Aim II

As stated above, there are three isoforms of the NOS enzyme and we have determined that iNOS is the principle NOS isoform that is involved in the MPTP neurotoxic process. Of note, is the fact that considerable iNOS expression has been found in the SNpc of post-mortem tissues from PD patients. iNOS upregulation has also been found in other situations where neurodegeneration occurs such as in Alzeheimer's disease and stroke), thus, the speculation that an inflammaroty (microglia involvement) situation may be part of the overall neurodegenerative process. Since iNOS is only minimally or not expressed at all in the brain under normal conditions and is up-regulated following MPTP administration, **Specific Aim II,** using the above-mentioned techniques, not only addressed the question of the main source of NO in the SNpc following MPTP administration to mice but also demonstrated which type of cell is instrumental in the production of NO. Microglia are the main source of NO in the MPTP neurotoxic process. Minocycline, a tetracycline can effectively attenuate the microglial response to MPTP administration in the brain.

Publications relevant to Specific Aim II.

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Specific Aim III

Peroxynitrite, a far more damaging species than either the superoxide radical or NO, is thought to be the result of the interaction between the two. For **Specific Aim III**, we have measured in selected brain regions of normal C57BL/6 mice (Charles River Laboratories) 3-nitrotyrosine, o,o-dityrosine and orthotyrosine following MPTP administration using gas chromatography with mass spectrometry. Having not only demonstrated that this is indeed feasible and that a number of MPTP-induced changes do occur, we are now breeding SOD1, nNOS and iNOS as well as the SOD crosses with nNOS and iNOS knockout mice for the measurements of 3-nitrotyrosine, o,o-dityrosine and orthotyrosine in these mice following MPTP administration. Breeding of these mice has to be done on a large scale as they will used for both **Specific Aims I and III.**

Publications Relevant to Specific Aim III.

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Specific Aim IV

The goals of **Specific Aim IV** are to assess the biological consequences of protein nitration by assessing whether candidate proteins such as manganese superoxide and mitochondrial electron transport chain polypeptide proteins as well as other proteins are nitrated. We have studied this in both in cell cultures exposed to peroxynitrite and in normal mice following MPTP administration. Thus far, not only is tyrosine hydroxylase, the rate-limiting enzyme in the production of catecholamines, nitrated, but other proteins such as α-synuclein, a presynaptic protein in both the ventral midbrain and the striatum, is also nitrated. Other candidate proteins like polypeptides from the mitochondrial electron transport chain and manganese superoxide are to be studied.

Publications Relevant to Specific Aim IV.

- Ara, J, Przedborski, S, Naini, AB, Jackson-Lewis, V, Trifiletti, RR, Horvitz, J, Ischiropoulos, H. Inactivation of tyrosine hydroxylase by nitration following exposure to peroxynitrite and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). PNAS (USA), 95, 7659-63, 1998.
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Abstracts Relevant to Specific Aim IV.

- Jackson-Lewis V, Neystat M, Lynch T, Vukosavic S, Burke RE, Przedborski S. Increased expression of α-synuclein in the MPTP mouse model of Parkinson.s disease (PD). 50th Annual meeting of the American Academy of Neurology, Minneapolis April 1998. Neurology 50, A97, 1998.
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Discussion and Conclusions.

Possessing a tool like MPTP that can replicate almost all of the hallmarks of PD and that has produced data pertinent to the oxidative stress hypothesis of PD has indeed been a gift. In following the salient points of our hypothesis concerning the involvement of the superoxide radical and NO and their interaction with one another in a pathological situation, we find that both the superoxide radical and NO (both reactive species [RS]) are increased following MPTP administration (5, 15,16). These findings are important in that they give teeth to the hypothesis that the oxidative stress hypothesis is indeed a heavy player in both PD and in the MPTP mouse model of PD. In our early work on this subject, using mice that overexpressed SOD, the enzyme that detoxifies the superoxide radical, we found that the SNpc in these mice was protected against the damaging effects of MPTP whereas normal the SNpc in normal mice not protected (5). From this, we concluded that the superoxide radical played a role in the MPTP neurotoxic process. During the course of the neurodegenerative process in the SN of MPTP-treated mice, we and others (15, 22, 23) noted a strong activation of microglia. The fact that there are three isoforms of the NO-producing enzyme, nitric oxide synthase (NOS) and the fact that we have demonstrated that blockade of nNOS upregulation by 7-nitroindazole in the SNpc of the MPTP-treated mouse model only partially protected SNpc DA neurons from MPTP (6) led us to believe that our hypothesis that other NOS isoforms might be involved in the MPTP neurotoxic process. Immunostaining methods as well as Western blot analyses

have proven this to be true as we have shown us that following MPTP administration, eNOS is not involved (personal communication) here but that iNOS apparently is. iNOS is induced in activated microglia and activated microglia are indicative of an inflammatory response (24-27). Our studies demonstrate a strong response from both microglia as well as from astroglia (15, 17) during the cell death phase following MPTP administration (28). This inflammatory response situation seems to promote the degeneration of the DA neurons in the SNpc following MPTP administration as other pro-inflammatory compounds such as IL-1β, certain cytokines and activation of several caspases have also been found in PD brains (29-32). The fact that activated microglia have been demonstrated in the post-mortem SNpc from PD brains and in the SNpc of human brains exposed to MPTP anywhere from three to sixteen years prior to death along with iNOS upregulation (33) strongly suggests that indeed we are on the right track. We were able to attenuation this microglial response following MPTP administration using the second generation tetracycline antibiotic minocycline (17).

Gliosis is a striking neuropathological feature in the SNpc and the striatum in the MPTP mouse model as in PD (15,17,22,23). However, activated microglia appear in the SNpc earlier than reactive astrocytes (15,17) and at a time when only minimal neuronal death occurs (28). This supports the contention that the microglial response to MPTP arises early enough in the neurodegenerative process to contribute to the demise of SNpc dopaminergic neurons. Consistent with this, is the demonstration that direct injection of the known microglial activator lipopolysaccharide into the rat SNpc causes a strong microglial response associated with significant dopaminergic neuronal death (34-36). Given these data, the key to the minocycline neuroprotective effect in the MPTP mouse model may lie in our finding that minocycline prevented the MPTP-induced microglial, but not astrocytic response in both the SNpc and the striatum. Inhibition of microglial activation using minocycline has already been demonstrated in other experimental models of brain insults (37-39) and results, presumably, from the blockade of P38 mitogen-activated protein kinase (37). It has been established that activated microglia can exert cytotoxic effects in the brain through two very different and yet complementary processes (40). First, they can act as phagocytes, which involves direct cell-to-cell contact and, second, they are capable of releasing a large variety of potentially noxious substances (40). Consistent with the notion that minocycline inhibits the ability of microglia to respond to injury, we show that minocycline not only prevents the microglial morphological response to MPTP, but also the microglial production of cytotoxic mediators such as IL-1β and the induction of critical ROS and NO producing enzymes such as NADPH-oxidase and iNOS (17). Little is known about the actual role of IL-1β in either MPTP or PD neurodegenerative process, except that IL-1β immunoreactivity is found in glial cells from post-mortem PD SNpc samples (33) and that blockade of interleukin converting enzyme, the known activator of IL-1β, attenuates MPTP-induced neurodegeneration in mice (42). As for ROS, oxidative stress is a prominent pathogenic hypothesis in both MPTP and PD (43,44). However, many of the microglial-derived ROS such as superoxide cannot readily transverse cellular membranes (43), making it unlikely that these extracellular reactive species gain access to dopaminergic neurons and trigger intraneuronal toxic events. Alternatively, superoxide can react with NO in the extracellular space to form the highly reactive tissue-damaging species, peroxynitrite,

which can cross the cell membrane and injure neurons. Therefore, microglial-derived superoxide, by contributing to peroxynitrite formation, may be significant in the MPTP model. As for NO in both MPTP and PD, the pivotal pathogenic role for microglial-derived NO is supported by the demonstration that ablation of iNOS attenuates SNpc dopaminergic neuronal death (15) and the production of ventral midbrain nitrotyrosine following MPTP administration (17). Remarkably, iNOS ablation does protect SNpc neurons from MPTP toxicity, but does not protect striatal nerve terminals and does not prevent microglial activation (15). This is in striking contrast to the effect of minocycline treatment which protects both dopaminergic cell bodies and nerve fibers and inhibits the entire microglial response (17) and strongly suggests that microglial-associated deleterious factors other than iNOS are involved in the demise of the nigrostriatal pathway in the MPTP mouse model of PD and possibly in PD itself.

Given that both the superoxide radical and NO are only mildly toxic to tissues, a stronger, more toxic compound is necessary to overwhelm the existing antioxidant protective systems in the brain. The theory that peroxynitrite might be the actual culprit is supported by our demonstration of the nitration and inactivation of tyrosine hydroxylase *in vitro* following exposure of HEK293 cells to peroxynitrite and *in vivo* following exposure of the mouse ventral midbrain to MPTP (19). Measurement of 3-nitrotyrosine was the indicator that tyrosine nitration had occurred (45). Nitrotyrosine is a stable fingerprint of NO-derived attack on protein which has been documented as one of the main markers of oxidative damage mediated by MPTP (46). Consistent with our previous studies (26,44), nitrotyrosine levels are consistently increased substantially in brain regions affected by MPTP, such as ventral midbrain, but not in brain regions unaffected by MPTP, such as cerebellum (15, 17). As with the loss of SNpc neurons and striatal fibers, minocycline dramatically attenuated ventral midbrain increases in nitrotyrosine levels (17). Collectively our data demonstrate that minocycline protects against morphological as well as biochemical abnormalities that arise from MPTP insult.

We demonstrated that α-synuclein is a specific target for tyrosine nitration in a cell model as well as in the mouse brain after MPTP challenge. First, non-transfected HEK293 cells and HEK293 cells overexpressing human α-synuclein or β-synuclein were exposed to peroxynitrite (46). This resulted in the nitration of a number of proteins as demonstrated by the Western blot analysis using an anti-nitrotyrosine antibody. However, only HEK293 cells transfected with α-synuclein showed a nitrated protein band with the molecular mass corresponding to α-synuclein. To demonstrate that α-synuclein was indeed nitrated, the total protein extract was subjected to immunoprecipitation using an anti-α-synuclein antibody and then the recovered immunoprecipitated protein was probed with the anti-3-nitrotyrosine antibody. This experiment confirmed that a significant fraction of the immunoprecipitated α-synuclein was definitely nitrated in the cells exposed to peroxynitrite, but not in untreated cells or in cells exposed to decomposed reagent.

Given these results, we then proceeded to assess whether α-synuclein was nitrated in the MPTP mouse model of PD. The use of this particular experimental model has been motivated by the fact that, thus far, significant insights into the pathogenesis of PD have

been achieved using this neurotoxin, which replicates in humans and in non-human primates a severe and irreversible PD-like syndrome, with concomitant degeneration of dopaminergic neurons (7). Moreover, several studies have indicated that reactive nitrogen species and tyrosine nitration not only occur in this model but also participate in the MPTP neurotoxic process (6, 9, 21, 47).

Immunoprecipitation of α-synuclein was performed in striatum and ventral midbrain, the two main targets of MPTP neurotoxicity (7). Immunoprecipitated α-synuclein from striatum and ventral midbrain, was selectively nitrated 4 hours after the MPTP challenge. Conversely, immunoprecipitation of α-synuclein from striatum and ventral midbrain of saline-injected mice, also at 4 hours post-injection did not reveal any detectable nitration of the protein. In contrast to the robust tyrosine nitration of α-synuclein, no tyrosine nitration was detected in two other pre-synaptic proteins, β-synuclein and synaptophysin, following a similar MPTP regimen. This observation is consistent with our previous finding that only selected proteins are tyrosine-nitrated after MPTP exposure (19, 46) and with the observation that α-synuclein but not ß-synuclein is nitrated after exposure of cells to the same peroxynitrite challenge. Moreover, this observation is consistent with the demonstrations that nitrated α-synuclein is present in the hallmark lesions in a number of human neurodegenerative synucleinopathies (48, 49, 50).

The higher efficiency of α-synuclein nitration is likely due to the unstructured conformation of the protein in aqueous solution, exposing all four tyrosine residues to solvent phase increasing the probability for the reaction with nitrating agents. Moreover, glutamate residues, a structural conformation associated with enhanced susceptibility of tyrosine to nitration, are near all three tyrosine residues 125, 133 and 136 in the carboxy terminal domain of α-synuclein. Indeed, purified human α-synuclein exposed to the nitrating agent *in vitro* and analyzed by electrospray mass spectrometry, revealed that nitration of α-synuclein occurs at all four tyrosine residues (51). In addition to nitration, exposure of α-synuclein to nitrating agents also results in the stable cross-linking of the protein via the formation of dityrosine (51). In contrast, ß-synuclein is nitrated to a lesser extent than α-synuclein *in vitro* and does not form stable *O-O'*dityrosine crosslinks after exposure to nitrating agents despite the presence of all four conserved tyrosine residues in both proteins (51). More significantly, we show here that in two *in vivo* models, α-synuclein is selectively nitrated, whereas nitration of β-synuclein is below detectable limits. The preferential nitration and oxidation of tyrosine residues in α-synuclein could be due to the accessibility of tyrosine residues to nitrating agents and by the presence of the protein in close proximity to the site(s) of generation of the nitrating agent. Our results raise the possibility that both syn proteins may have different confirmations or that β-synuclein may be protected from oxidation perhaps by different interacting partners *in vivo*. We have previously argued that proximity to sites of superoxide generation may be important in determining proteins modified by nitration as overexpression of superoxide dismutase and superoxide mimetics have been shown to prevent the nitration of proteins *in vivo* and in cell models (19, 52, 53).

The significance of the tyrosine nitration of α-synuclein remains unclear. Tyrosine nitration induces secondary and tertiary structural alterations, which may critically

modify protein functions (45). The change in the ionization state of the modified protein induced by a local shift in the pKa from 10.01 of tyrosine to 7.5 of 3-nitrotyrosine and the consequent changes in hydrophobicity and conformation may facilitate interactions with other proteins, promoting protein aggregation. Preliminary data indeed indicate an increased adherence of brain mouse extracts to nitrated α-synuclein compared to the unmodified wild type protein (Chen et al. unpublished observation). Collectively, the data indicate that α-synuclein is a preferential target for oxidative stress-mediated post-translational modifications. These alterations may trigger abnormal protein compartmentalization and aggregation, two phenomena that are potential culprits for the neurodegeneration process in PD. Thus, our evidence for the involvement of the superoxide radical and NO in the degeneration of SNpc DA neurons and the far-reacjing consequences thereof are germaine to the oxidative stress theory of PD and to the neurotoxic effects of MPTP.

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Inactivation of tyrosine hydroxylase by nitration following exposure to peroxynitrite and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

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ABSTRACT **The decrement in dopamine levels exceeds the loss of dopaminergic neurons in Parkinson's disease (PD) patients and experimental models of PD. This discrepancy is poorly understood and may represent an important event in the pathogenesis of PD. Herein, we report that the rate-limiting enzyme in dopamine synthesis, tyrosine hydroxylase (TH), is a selective target for nitration following exposure of PC12 cells to either peroxynitrite or 1-methyl-4-phenylpyridiniun ion (MPP^+). Nitration of TH also occurs in mouse striatum after MPTP administration. Nitration of tyrosine residues in TH results in loss of enzymatic activity. In the mouse striatum, tyrosine nitration-mediated loss in TH activity parallels the decline in dopamine levels whereas the levels of TH protein remain unchanged for the first 6 hr post MPTP injection. Striatal TH was not nitrated in mice overexpressing copper/zinc superoxide dismutase after MPTP administration, supporting a critical role for superoxide in TH tyrosine nitration. These results indicate that tyrosine nitration-induced TH inactivation and consequently dopamine synthesis failure, represents an early and thus far unidentified biochemical event in MPTP neurotoxic process. The resemblance of the MPTP model with PD suggests that a similar phenomenon may occur in PD, influencing the severity of parkisonian symptoms.**

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by disabling motor abnormalities attributed to a profound deficit in dopamine (1). The decline in dopamine level has been thought to arise solely from the severe loss of dopaminergic neurons in the nigrostriatal pathway. However, the dopamine deficit in the affected regions of the brain significantly exceed the loss of dopaminergic neurons (2, 3), suggesting that dopamine synthesis is impaired before cellular demise. Support for this hypothesis comes from studies of experimental models of PD demonstrating that the reduction in dopamine metabolism-related markers such as tyrosine hydroxylase (TH) and dopamine transporter is far greater than the loss of neuronal cell bodies (4–6). Because the severity of PD symptoms correlates with the magnitude of dopamine deficit, elucidating mechanisms that impair dopamine synthesis and metabolism in neurons that undergo selective degeneration in PD may have important therapeutic implications.

There is experimental evidence from studies of humans and animals in support of the hypothesis that oxidative stress contributes to the pathogenesis of PD (7). Studies performed in the MPTP model of PD suggest that peroxynitrite, a reactive species formed by the nearly diffusion-limited reaction of nitric oxide with superoxide, may be a mediator of nigrostriatal damage in PD (8–10). The potential role of peroxynitrite in the pathogenesis of PD is further supported by demonstrating that exposure of the monoamine-producing PC12 cells to peroxynitrite induced a dose-dependent alteration in dopamine synthesis that was not due to cell death or the oxidation of dopamine (11).

Based on these observations, we propose that the inhibition of dopamine metabolism in PD may result from the peroxynitrite-mediated inactivation of TH, the rate limiting enzyme in dopamine synthesis. Previous work has shown that protein tyrosine residues are a major target of peroxynitrite reactivity. The nitration of the ortho position of tyrosine by peroxynitrite occurs spontaneously as well as by CO_2 or low molecular mass transitional metal catalysis (12–14). The latter mechanism may be particularly relevant to PD because the free iron content in affected brain regions is markedly increased (15, 16). Protein associated or free nitrotyrosine has been detected in human postmortem specimens of patients with neurodegenerative disorders such as Alzheimer's, multiple sclerosis, and amyotrophic lateral sclerosis, as well as in animal models of neurodegeneration (17–23).

Therefore, experiments were performed to test the hypothesis that the inactivation of dopamine synthesis is caused by nitration of TH by peroxynitrite in models of PD. Nitration of TH was examined in PC12 cells challenged with different concentrations of peroxynitrite and 1-methyl-4-phenylpyridinium ion (MPP^+), the active metabolite of MPTP (24), as well as in mice treated with MPTP. Nitration of TH was detected and quantified by immunoprecipitation and reaction with affinity purified anti-nitrotyrosine antibodies and amino acid analysis. Nitrated TH was found in all models, and the extent of TH nitration correlated with loss of TH catalytic activity and decline in dopamine levels.

MATERIALS AND METHODS

Exposure of PC12 Cells and Tyrosine Hydroxylase to Peroxynitrite. Peroxynitrite was synthesized from nitrite and hydrogen peroxide. The concentration of peroxynitrite was measured by the increase in absorbance at 302 nm ($\varepsilon_{302\ nm}$ = 1,700 $M^{-1}\ cm^{-1}$) in 1.2 M NaOH. The peroxynitrite was added to the samples as a small drop along the wall of the tube just above the reaction mixture and then rapidly mixed by vortex-

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Abbreviations: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; TH, tyrosine hydroxylase; MPP^+, 1-methyl-4-phenylpyridiniun ion; Cu/Zn, copper/zinc; SOD, superoxide dismutase.

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ing. The pH of the buffer was the same after each addition of peroxynitrite. PC12 cells were washed with Earle's balanced salt solution, scraped off the plates, and centrifuged at 800 × *g* for 5 min, and the pellet was solubilized with lysis buffer (20 mM Tris·HCl, pH 7.4/150 mM NaCl/4 mM EGTA/10% glycerol/1% Triton X-100) (Bio-Rad).

Immunoprecipitation of Tyrosine Hydroxylase and Nitrotyrosine Detection. Mice brain homogenates and PC12 cell lysates were precleared with protein G-Sepharose (Pharmacia) (1 hr at 4°C) to reduce the amount of protein precipitated nonspecifically. The mixture was centrifuged for 1 min at 10,000 rpm to pellet the beads with nonspecifically bound proteins. Five microliters of anti-tyrosine hydroxylase mAbs (1 mg/ml) were incubated for 12 hr at 4°C with 500 μl of appropriately diluted samples in lysis buffer containing 1 mM PMSF, 10 mg/ml aprotinin, 0.2 mM sodium orthovanidate, and 1 mg/ml leupeptin. The immune complexes were precipitated with 30 μl of 25% wt/vol protein G-Sepharose (rotating the suspension for 1 hr and 30 min at 4°C) after which the beads were collected by centrifugation and washed three times with lysis buffer. The beads were finally suspended in 50 μl of sample buffer containing SDS and 2-mercaptoethanol and heated at >90°C for 5–10 min. The protein G-Sepharose was pelleted by centrifugation for 1 min, and supernatant was analyzed by SDS gel electrophoresis on 12% running gels. Proteins were transferred to 0.2-mm nitrocellulose membranes (Schleicher & Schull) and reacted with either a polyclonal anti-tyrosine hydroxylase 1:3,000 dilution (Eugene Tech, Ridgefield, NJ) or 1.0 μg/ml affinity purified rabbit anti-nitrotyrosine antibodies that were preconjugated overnight at 4°C with 1:3,000 dilution of horseradish peroxidase labeled-goat anti-rabbit IgG (H+L; Bio-Rad). After washing, the nitrocellulose was incubated with chemiluminescent substrate (Amersham) and then exposed to the x-ray film (AIF, Fuji).

Animals and Treatment. Eight-week-old male C57/bl mice (25–30 g; Charles River Breeding Laboratories) were housed three per cage in a temperature-controlled room under a 12-hr light/dark cycle with free access to food and water. On the day of the experiment, mice received four i.p. injections of MPTP·HCl (20 mg/kg free base; Research Biochemicals) in saline at 2 hr intervals; control mice received saline only. MPTP-injected mice (4–6 per group) were killed 0, 3, and 6 hr after the last injection. Right and left striata were rapidly dissected on ice, immediately frozen on dry ice, and stored at −80°C until analysis (3). To examine the effects of copper/zinc superoxide dismutase (SOD1) activity on tyrosine nitration of the enzyme tyrosine hydroxylase, hemizygote male (aged 2–8 mo) SF-218 mice also were injected with MPTP as above. These mice carry eight copies of the wild-type human SOD1 gene, presumably in tandem array, and have ≈fourfold higher striatal and ventral midbrain SOD1 activity compared with their nontransgenic littermates (25).

Measurement of Striatal Dopamine Levels. HPLC with electrochemical detection was used to measure striatal levels of dopamine. On the day of the assay, frozen tissue samples were sonicated in 50 vol (wt/vol) of 0.1 M perchloric acid containing 25 ng/ml dihydrobenzylamine (Sigma) as internal standard. After centrifugation (15,000 × *g*, 10 min, 4°C), 20 μl of supernatant was injected onto a C18-reversed phase RP-80 catecholamine column (ESA, Bedford, MA). The mobile phase consisted of 90% of a solution of 50 mM sodium phosphate, 0.2 mM EDTA, and 1.2 mM heptanesulfonic acid (pH 3.5), and 10% methanol. Flow rate was 1.0 ml/min. Peaks were detected by a Coulochem 5100A detector (E1 = −0.04 V, E2 = +0.35 V) (ESA). Data were collected and processed on a computerized Dinamax data manager (Rainin, Woburn, MA).

Tyrosine Hydroxylase Activity. A radiometric assay based on the release of $[^3H]H_2O$ from L-[ring-3,5-^{3}H]tyrosine (NEN) was used to determine the striatal catalytic activity of tyrosine hydroxylase (26). Frozen samples were sonicated in 10 vol (wt/vol) 50 mM Mes (pH 6.1) and centrifugated (15,000 × *g*, 10 min, 4°C). In a total volume of 100 μl, 50 μl of supernatant were mixed with 25 μl of a mixture containing 2.5 nmol L-tyrosine, 1 mCi (1 Ci = 37 GBq) L-[ring-3,5-^{3}H]tyrosine (specific activity 50 Ci/mmol, NEN), 0.5 mmol DTT, and 15 μl catalase (80 units/ml). The reaction was started by adding 10 μl of 10 mM 6(R)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH_4, Sigma); for blank, BH_4 was omitted and 10 μl of Mes buffer was added instead. At the end of the incubation (20 min, 37°C), 1 ml of cold 7.5% activated charcoal (R-60, Fisher Scientific) suspension in 1 M HCl was added to each tube, vortexed, and centrifuged (2,000 × *g*, 5 min, 25°C). Then, 0.4 ml of the supernatant was mixed with 4 ml of Acquasol-II (NEN), and radioactivity was counted by scintillation spectrometry. Protein concentrations were determined by using the Bradford assay (Pierce).

Tyrosine Hydroxylase Protein. An ELISA was used to measure striatal content of tyrosine hydroxylase protein according to the method reported by Reinhard, Jr. *et al.* (27) with minor modifications. Falcon 96-well polystyrene ELISA plates (Fisher Scientific) were coated with 10 ng/well of rabbit polyclonal anti-tyrosine hydroxylase (Gift from J. W. Haycock, Louisiana State University Medical Center, New Orleans) in 10 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl (PBS) by overnight incubation at 4°C. The plates were then washed (PBS containing 0.05% Tween 20), blocked with 1% BSA in PBS (1 hr, 25°C), and stored at 4°C. On the day of the assay, frozen samples were homogenized (glass/glass homogenizer) in 10 vol (wt/vol) PBS containing 1 mg/ml leupeptin (Sigma). After centrifugation (15,000 × *g*, 10 min, 4°C), 100 μl of supernatant diluted at 1:75 in buffer were added to the well and incubated (30 min, 37°C); for blanks, 100 μl of buffer were added instead of supernatant. The plates were successively incubated with 1:16,000 mouse monoclonal anti-tyrosine hydroxylase (TH-16, Sigma), 1:400 biotinylated anti-mouse IgG (Vector, Burlingame, CA), and 1:200 horseradish peroxidase conjugated-streptavidin (Vector) in PBS; between each of these incubations (30 min, 37°C), plates were washed twice with PBS/0.05% Tween 20 at 25°C. After the last wash, 100 μl of horseradish peroxidase substrate 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Vector) was added to each well and the plate was incubated (20 min, 25°C) in the dark before being read at 405 nm (with reference at 450 nm) on a computerized dual microplate reader (Bio-Rad model 3550). Tyrosine hydroxylase content (μg/mg protein) was derived from a purified rat tyrosine hydroxylase standard curve with a linear concentration range of 1.25–20 ng/well.

Two-Dimentional Electropheresis. Brain tissue was dissected and sonicated immediately in ice-cold PBS (pH 7.4), and the sonicate was centrifuged at 13,000 × *g* for 20 min. The supernatant obtained was either analyzed immediately or stored at −70°C; the pellet was resolubilized in 10 vol PBS and stored at −70°C. Proteins (100 μg/tube) were first separated by isoelectric focusing in capillary tubes by using pH 3–10 ampholytes (Bio-Rad). Denaturing SDS/PAGE was performed on miniature 8% polyacrylamide gels enabling separation of proteins with apparent molecular mass of 5–200 kDa. Color molecular weight markers (Sigma) were run in all gels, enabling visual verification that the membrane transfer was complete. To calibrate the pH, parallel runs were performed by using pI markers (Sigma). Before staining, blots were incubated overnight at 4°C in a 5% blocking solution (nonfat dry milk in PBS, pH 7.4). Blots were then washed three times for 5 min/wash in PBS containing 0.05% Tween-20 and a 5-min wash in PBS. Blots were incubated at 25°C in a primary antibody solution of 1 μg/ml anti-nitrotyrosine antibody in 3% blocking buffer. Blots were then washed and incubated 1 hr in biotinylated secondary antibody (goat anti-rabbit IgG; Sigma) in 3% blocking buffer. Blots were then washed and incubated

30 min in streptavidin–biotin-linked horseradish peroxidase solution and visualized with chemiluminescence as described above. Appropriate controls showed that all staining can be eliminated by either elimination of the primary antibody or by including 10 mM 3-nitrotyrosine in the initial primary antibody solution (28).

RESULTS

PC12 Cell Models. PC12 cell lysates rich in TH were treated with different concentrations of peroxynitrite, and immunoprecipitated TH was reacted with affinity purified anti-nitrotyrosine antibodies. As expected, exposure to peroxynitrite resulted in a dose-dependent nitration of TH (Fig. 1*A*). The degree of TH nitration correlated with the loss of enzymatic activity (Fig. 1*B*). TH activity was assayed under optimal conditions to insure that the decline in activity is not related to availability of substrate or cofactor but to TH modification(s). Other amino acid residues susceptible to peroxynitrite attack are tryptophan, cysteine, and methionine. The content of tryptophan in purified TH was determined by measuring the fluorescence of the indole ring (excitation λ = 325 nm and emission λ = 410 nm). Rat and human TH contains three tryptophan residues that are sufficient to permit accurate detection of fluorescence. There was no difference in the fluorescence of the indole ring of unreacted purified TH and TH reacted with up to 1 mM peroxynitrite. Amino acid analysis of purified TH before and after reaction with peroxynitrite did not reveal any other change in the recovery of amino acids including methionine. Therefore, in the absence of any other amino acid modification, tyrosine nitration is the primary reason for the inactivation of the enzyme. Moreover, amino acid analysis of purified TH exposed to peroxynitrite revealed that the same mol percentage of nitrotyrosine (0.32 ± 0.03) was present irrespective of the peroxynitrite concentration. Exposure of purified TH to 2 mM peroxynitrite results in nitration of all TH molecules, and thus under this condition, the 0.32 mol percent represents nitration of a single tyrosine residue in every TH molecule.

PC12 cells also were exposed for 1 hr to different concentrations of MPP^+. In similar to exposure to peroxynitrite, MPP^+ induced the nitration of TH (Fig. 2). It is important to note that the PC12 cells used in these experiments contain the neuronal form of nitric oxide synthase (nNOS), documented

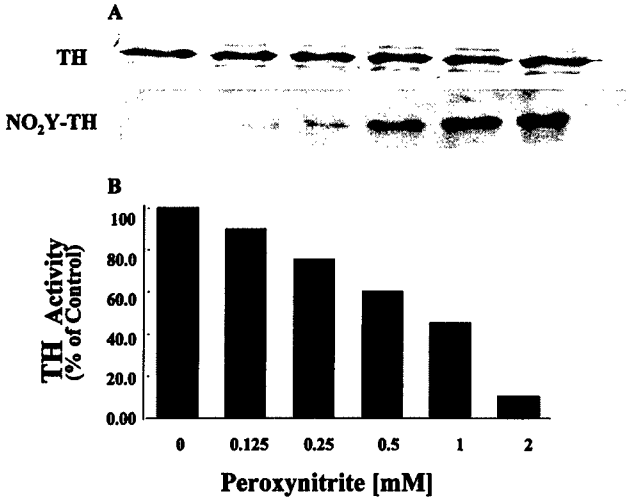

FIG. 1. Nitration of TH molecules results in proportional loss of activity. (*A*) Levels of immunoprecipitated TH in PC12 cell lysates treated with different concentrations of peroxynitirite. (*B*) Nitrated TH. The level of nitration was evaluated after staining with affinity purified polyclonal anti-nitrotyrosine antibodies and by amino acid analysis. (*C*) TH activity determined under optimal conditions.

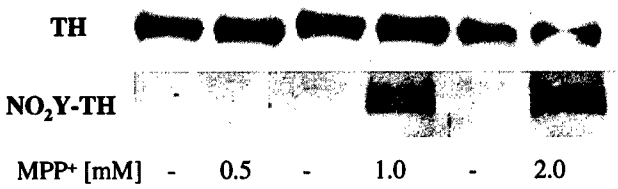

FIG. 2. (*A*) Immunoprecipitation of TH after 1-hr treatment of PC12 cells with different concentrations of MPP^+. (*B*) Nitration of tyrosine residues in the immunoprecipitated TH was evaluated by reaction with the anti-nitrotyrosine antibodies. Representative data from three independent experiments.

by Western blot analysis (not shown) and by the presence of nitric oxide metabolites, nitrite and nitrate. Under our experimental conditions, lysates of 1×10^6 cells contain 3.7 ± 0.4 μM nitrite plus nitrate. Moreover, 1 hr after exposure to MPP^+, the cells were viable and capable of reducing 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to formazan. Control cells value for MTT reduction was 0.800 ± 0.07 absorbance at 570 nm, (n = 3, mean ± SD) and of cell treated with 1 mM MPP^+, 0.979 ± 0.04 absorbance at 570 nm, (n = 3 mean ± SD). The reduction of MTT by the mitochondrial and cytosolic dehydrogenase(s) requires NADH and, therefore, reflects the availability of pyridine nucleotides and the cellular redox state.

MPTP Murine Models. To determine whether MPTP induces nitration of TH *in vivo*, adult C57/bl mice were injected with MPTP and controls were injected with saline as described (8). In both striatum and ventral midbrain, increases in protein tyrosine nitration were detected as early as 3 hr after MPTP injection. Two-dimensional separation of proteins followed by immunoblotting with anti-nitrotyrosine antibody revealed that several proteins were nitrated in the striatum 3 hr after MPTP injection (Fig. 3). A prominent nitrated band was a protein with an apparent molecular weight of 58,000 and pI of 5.8, which are physical properties of tyrosine hydroxylase (29–31). To confirm that TH is the nitrated protein, TH from striatal protein extracts of MPTP-injected mice was immunoprecipitated and subsequently reacted with affinity purified anti-nitrotyrosine antibodies. Striatal TH was indeed nitrated 3 and 6 hr after MPTP injection (Fig. 4*A*) whereas TH immunoprecipitated from saline controls or immediately after the last injection of MPTP was not nitrated. To demonstrate the critical role of superoxide in tyrosine nitration of TH, MPTP was administrated to transgenic mice with increased SOD1 activity, the enzyme responsible for the conversion of cytosolic superoxide to hydrogen peroxide. In the transgenic mice with increased SOD1 activity, MPTP did not cause any detectable nitration of striatal TH at any time points studied (Fig. 4*B*).

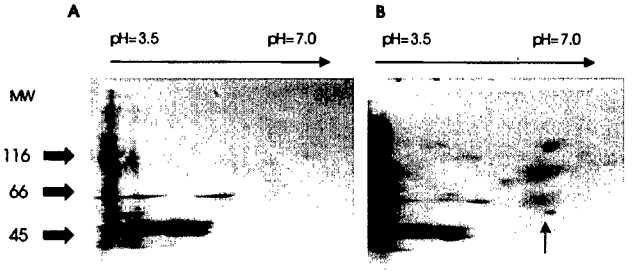

FIG. 3. MPTP treatment leads to protein tyrosine nitration of specific proteins in the mouse midbrain. Two-dimensional, chemiluminescence-enhanced anti-nitrotyrosine immunoblots of ventral midbrain tissue of C57/bl mice 3 hr after injection of either saline (*A*) or MPTP (*B*). MPTP injection resulted in nitration of selective proteins. Indicated by the arrow is a protein with the physical characteristics of mouse tyrosine hydroxylase listed in protein databases.

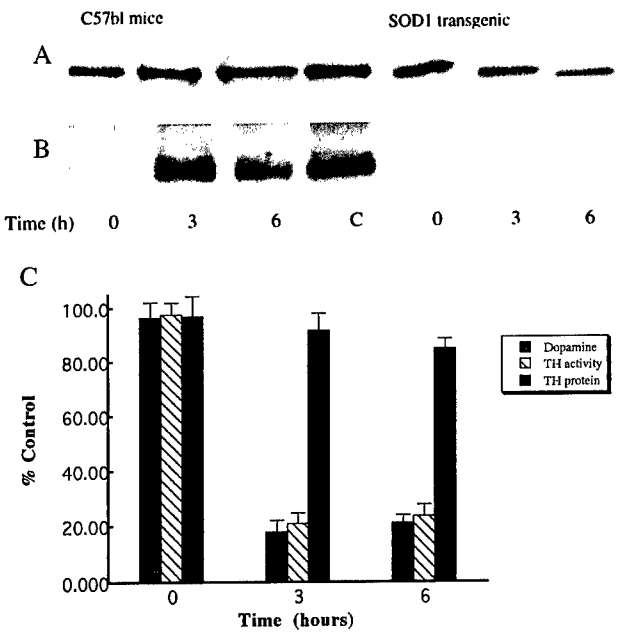

FIG. 4. (*A*) Immunoprecipitation of striatal TH from MPTP-exposed mice immediately after exposure (time 0), 3- and 6-hr post exposure. TH was visualized with polyclonal anti-tyrosine hydorxylase antibodies. Representative data from four different striatal preparations. (*B*) Nitration of tyrosine residues in the immunoprecipitated TH was evaluated by reaction with the anti-nitrotyrosine antibodies. As a positive control (C), TH was laso immunoprecipitated from peroxynitrite treated PC12 cell lysates as described in Fig. 1. (*C*) Time course of striatal dopamine, tyrosine hydroxylase activity and TH protein levels after MPTP administration (n = 4–6).

This observation is consistent with the previous reports that MPTP exposure of SOD1-transgenic mice failed to induce a dopamine deficit (25).

The present regimen of MPTP caused dramatic reductions in striatal dopamine levels in C57/bl mice similar to previously reported albeit at earlier time points (4, 8, 25). Striatal dopamine levels declined to nearly 20% of saline control 3 and 6 hr post-injection. The magnitude and the time course of the changes in striatal TH activity following MPTP administration paralleled very closely that of dopamine levels (Fig. 4*C*). Conversely, loss in striatal TH protein content was insignificant (only 6% as compared with zero time) during the first 6 hr post MPTP injection.

DISCUSSION

Previously we demonstrated that peroxynitrite impairs dopamine metabolism in PC12 cells (11). In the present study, the molecular mechanism for the peroxynitrite-impairment of dopamine production and the potential role of this process in the pathogenesis of MPTP model of PD was investigated. The rate-limiting enzyme in the dopamine synthesis, TH, is a target for peroxynitrite-induced tyrosine nitration and that nitration of a single tyrosine residue within this enzyme appears to be sufficient to impair its catalytic activity. Nitration of tyrosine residues *in vitro* results in inactivation of a vast number of mammalian proteins whose activity is dependent on tyrosine residues (32). Nitration of tyrosine results in a marked shift of the local pK_a from 10.07 of the OH group of tyrosine to 7.5 of 3-nitrotyrosine that is expected to change the hydrophobicity, hydrogen bonding, and electrostatic interactions within the protein. The shift in pK_a provided the biochemical explanation for the inhibition in the rate of phosphorylation by tyrosine kinases as well as for the inactivation of protein function (28). Native tyrosine 3-hydroxylase (EC 1.14.16.2) is a tetrameter of four identical monomers. Isolated monomers have catalytic activity, and by limited proteolytic digestion, the catalytic domain has been located in the carboxyl-terminal between residues Leu188 and Phe456 (29–31). The rat enzyme contains 17 tyrosine residues of 498 total residues, and 15 of these tyrosine residues are found in the catalytic domain (30, 31). The human enzyme contains 15 tyrosine residues with 14 of them in the catalytic domain. Although the site of nitration has not been identified, Tyr225 in both the rat and human TH is a likely candidate because it is located within a sequence (X-X-Glu-Tyr-Thr-Ala) that is a target for nitration by peroxynitrite. This sequence was found to be the most effective target for tyrosine nitration during a screening of peptide sequences for efficiency of nitration by peroxynitrite (H.I., unpublished results).

The pivotal role of TH in catechdamine synthesis, and consequently in PD, led to the second set of experiments aimed at assessing whether MPTP that is suspected to exert its neurotoxic effects, at least in part, through the formation of peroxynitrite, also would inactivate TH. MPTP produced a rapid and profound loss in striatal dopamine content that was closely matched by the loss in TH activity. The loss in TH activity was not caused by a decrease in striatum TH protein. In addition, negligible dopaminergic neuronal death is observed 6 hr after MPTP injection (4). Therefore, TH inactivation and dopamine synthesis failure is an early event in MPTP neurotoxic process that precedes loss in TH protein and dopaminergic neurons. Nagatsu and coworkers (33, 34) have found at least three distinct phases in TH activity and content after either MPTP or MPP^+ administration. During the early phase, minutes after MPTP or MPP^+ administration there is a decrease in the *in vivo* TH activity but not in the V_{max} of the enzyme measured under optimal conditions *in vitro*. During the same time, the TH concentration remains the same, and as a result the homeospecific activity of TH, expressed as V_{max} over TH protein, also remains the same. The loss of TH activity *in vivo* was attributed to either the increase in cytoplasmic levels of dopamine or to the inhibition of TH phoshorylation (34, 35). During the middle phase, such as 3 hr after continuous exposure of striatal dopaminergic neurons to MPP^+ or after the last injection of MPTP, the TH activity both *in vivo* and *in vitro* was decreased significantly whereas the TH protein levels are unchanged (33, 34). As expected, the decrease in the V_{max} resulted in a decrease in the homeospecific activity of TH. The mechanism for the inactivation of TH and loss of homeospecific activity during the middle phase (hours) has not been elucidated. This study provides evidence that the molecular mechanism responsible for the inactivation of TH after MPTP administration is tyrosine nitration. Previously, it was shown that MPTP stimulates nitration of free tyrosine (10) and of total proteins (22), but the current work indicates that TH is a preferential target for MPTP-induced tyrosine nitration. The late phase of MPTP or MPP^+ administration resembles the late stages of PD disease in which TH activity *in vivo* and *in vitro*, TH protein content are significantly decreased (33, 34). In PD patients, a compensatory increase in TH-homeospecific activity has been reported and is thought to arise from the existence of multiple forms of TH mRNA, which are products of alternative splicing (35).

The existence of nitrated TH implies the formation of a nitrating agent after injection of MPTP. MPTP has been shown to selectively accumulate in dopaminergic neurons where it is oxidized to MPP^+ by monoamine oxidase-B (24). In turn, redox cycling of MPP^+ by dehydrogynases such as the complex I of the mitochondrial electron transport chain and cytosolic oxidoreductases such as the cytochrome P450 reductase and xanthine oxidase results in the formation of superoxide by the one electron reduction of oxygen (36). Thus, in both the mitochondria and cytosol compartments, the redox cycling of MPP^+ results in an increase in the steady–state levels

of superoxide. We hypothesize that the increase in the steady-state levels of superoxide in dopaminergic neurons allows for the formation of peroxynitrite because the second order rate constant for the reaction of superoxide with nitric oxide is $0.4–1 \times 10^{10}$ M^{-1} s^{-1}, which is essentially the fastest reaction known for superoxide to date (37). Nitric oxide is membrane-permeable and can diffuse into dopaminergic neurons. Thus, it is the site of elevated levels of superoxide that determines whether a cell will be affected by the reactivity of peroxynitrite. Although peroxynitrite is a strong oxidant, it reacts with most biological targets with relatively slow rates (second order rate constants range from 10^3 to 10^6 M^{-1} s^{-1}) (38). Therefore, the concentration of the biological target and the proximity to the formation of peroxynitrite will determine which molecule will react with peroxynitrite. It is apparent from data in this study and in other models that protein tyrosine residues are selective targets for peroxynitrite resulting in nitration. In the MPTP model of PD, TH appears to be a major and critical target for peroxynitrite-mediated nitration that leads to enzymatic inactivation. As such, this finding represents the first example of *in vivo* protein nitration that leads to a functional deficit that is directly related with a pathogenic outcome.

Support for our working hypothesis also comes from transgenic mice with increased SOD1 activity. The decrease in the steady-state levels of superoxide and consequently of peroxynitrite was able to prevent nitration of TH and loss of enzymatic activity. Therefore the previous findings that these mice are resistant to MPTP neurotoxicity can now be biochemically explained on the basis of preventing peroxynitrite formation. Blocking the formation or effective scavenging of peroxynitrite can potentially provide a therapeutic intervention because it will eliminate nitration of TH and loss of catalytic activity preserving dopamine levels in the brain. Moreover, eliminating peroxynitrite also may be useful because this reactive species can induce apoptosis and delayed cell death independent of its nitrating ability (39–43).

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The parkinsonian toxin MPTP: action and mechanism

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Abstract

MPTP causes damage to substantia nigra pars compacta (SNpc) dopaminergic (DA) neurons as seen in Parkinson's disease (PD). After systemic administration of MPTP, its active metabolite, MPP^+, accumulates within SNpc DA neurons, where it inhibits ATP production and stimulates superoxide radical formation. The produced superoxide radicals react with nitric oxide (NO) to produce peroxynitrite, a highly reactive tissue-damaging species that damages proteins by oxidation and nitration. Only selected proteins appear nitrated, and among these, is found tyrosine hydroxylase (TH), the rate limiting enzyme in DA synthesis. The process of nitration inactivates TH and, consequently dopamine production. Peroxynitrite also nicks DNA, which, in turn, activates poly(ADP-ribose) polymerase (PARP). PARP activation consumes ATP, and thus acutely depletes cell energy stores. This latter event aggravates the preexisting energy failure due to MPP^+-induced mitochondrial respiration blockade and precipitates cell death. Altogether, these findings support the view that MPTP's deleterious cascade of events include mitochondrial respiration deficit, oxidative stress, and energy failure. Because of the similarity between the MPTP mouse model and PD, it is tempting to propose that a similar scenario applies to the pathogenesis of PD.

Keywords: Free radicals, MPTP, neurodegeneration, NO, Parkinson's disease, poly(ADP-ribose) polymerase, tyrosine hydroxylase

1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder of unknown cause whose cardinal clinical features include shaking, stiffness, slowness of movement, and postural instability [20]. Most, if not all, of these disabling clinical abnormalities are attributed to a profound decrease in dopamine content in the striatum which results from the dramatic loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) [20]. The prevalence of PD has been estimated at ~1,000,000 in North America with ~50,000 newly affected individuals each year. Thus far, the most potent treatment for PD remains the administration of a precursor of dopamine, L-DOPA, which, by replenishing the brain with dopamine, alleviates PD symptoms. However, the chronic administration of L-DOPA often causes motor and psychiatric side effects, which may be as debilitating as PD itself [37]. Furthermore, there is no supportive evidence that L-DOPA therapy impedes the progressive death of SNpc dopaminergic neurons. Therefore, without undermining the importance of L-DOPA therapy in PD, it remains essential to elucidate the cascade of events that underlie PD's neurodegenerative process. To this end and in light of the rarity of available post-mortem brain samples from PD patients and the fact that autopsy materials essentially represent end-stage PD, many investigators, including ourselves, have focused their research efforts on experimental models of PD such as the one produced by the parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).

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2. MPTP – a model of Parkinson's disease

The fact that MPTP causes a parkinsonian syndrome was discovered in 1982 when a group of drug addicts in California were rushed to the emergency room with a severe bradykinetic and rigid syndrome [40]. Subsequently, thanks to some fine detective work, it was discovered that this syndrome was induced by the self-administration of street batches of a synthetic heroin analogue whose synthesis had been heavily contaminated by a by-product, MPTP [41]. In the period of a few days following the administration of MPTP, these patients exhibited a severe and irreversible akinetic rigid syndrome. The analogy to PD was rapidly made by Dr. Langston and his group, and levodopa was tried with great success, relieving the symptoms of these unfortunate patients.

Since the discovery that MPTP causes parkinsonism in human and non-human primates as well as in various other mammalian species, this neurotoxin has been used extensively as a model of PD [26,36,41]. In human and non-human primates, MPTP produces an irreversible and severe parkinsonian syndrome that replicates almost all of the features of PD including tremor, rigidity, slowness of movement, postural instability, and even gait freezing. The responses as well as the complications to traditional anti-parkinsonian therapies are virtually identical to those seen in PD. However, while in PD, it is believed that the neurodegenerative process occurs over several years, MPTP produces a clinical condition consistent with "end-stage PD" in a few days [39]. Except for a single case [16], no human pathological material has been available. Thus, the comparison between PD and the MPTP model is largely limited to primates [21]. From neuropathological data, MPTP administration causes damage to the dopaminergic pathways identical to that seen in PD [1] with a resemblance that goes beyond the degeneration of SNpc dopaminergic neurons. Like PD, MPTP causes a greater loss of dopaminergic neurons in the SNpc than in the ventral tegmental area [53,67] and a greater degeneration of dopaminergic nerve terminals in the putamen than in the caudate nucleus [52]. On the other hand, two typical neuropathologic features of PD have, until now, been lacking in the MPTP model. First, except for the SNpc, the other pigmented nuclei such as the locus coeruleus have been spared according to most published reports. Second, the eosinophilic intraneuronal inclusions, called Lewy bodies, so characteristic of PD, have thus far not been convincingly observed in MPTP-induced parkinsonism [21]. Also worth noting is the fact that post-mortem brain samples from PD patients [18] show a selective defect in the same mitochondrial electron transport chain complex that is affected by MPTP [22,56]. Abnormalities in parameters of oxidative stress in post-mortem PD brain tissue suggest that this disease is caused by an overproduction of free radicals [58], the same highly reactive tissue damaging species that are suspected of being involved in MPTP-induced dopaminergic toxicity *in vivo* [24,61,66]. However, despite this impressive resemblance between PD and the MPTP model, MPTP has never been recovered from post-mortem brain samples or body fluids of PD patients. Altogether, these findings are consistent with MPTP not causing PD, but being an excellent experimental model of PD. Accordingly, it can be speculated that elucidating the molecular mechanisms of MPTP should lead to important insights into the pathogenesis and treatment of PD.

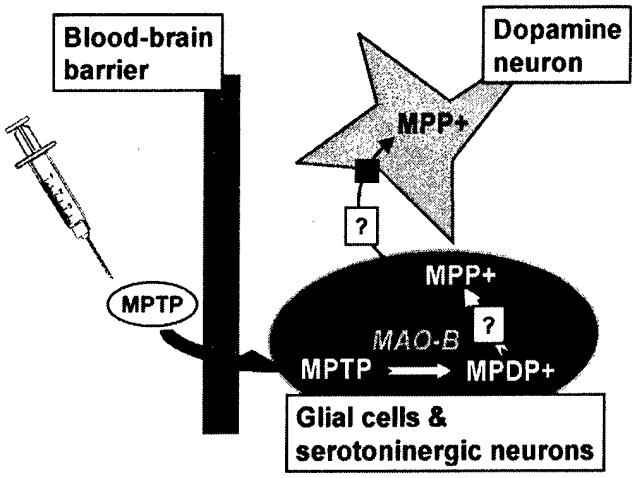

Fig. 1. Schematic representation of MPTP metabolism. After its systemic administration, MPTP crosses the blood-brain barrier. Once in the brain, MPTP is converted to $MPDP^+$ by MAO-B within non-dopaminergic cells, and then to MPP^+ by an unknown mechanism (?). Thereafter, MPP^+ is released, again by an unknown mechanism (?), in the extracellular space. From there, MPP^+ is taken up by the DAT and thus enter dopaminergic neurons.

3. Mode of action of MPTP

As illustrated in Fig. 1, the metabolism of MPTP is a complex, multistep process [59]. After its systemic administration, MPTP, which is highly lypophilic, rapidly crosses the blood-brain barrier. Once in the brain, the pro-toxin MPTP is metabolized to 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) by the enzyme monoamine oxidase B (MAO-B) within non-dopaminergic cells, and then (probably by spontaneous oxidation) to 1-methyl-4-phenylpyridinium (MPP^+), the active toxic compound. Thereafter, MPP^+ is released (by an unknown mechanism) in the extracellular space. Brain inflow of MPTP, together with its transformation into MPP^+, determine the amount of MPP^+ available to enter dopaminergic neurons. The next important step in the MPTP neurotoxic pathway is the mandatory entry of MPP^+ into dopaminergic neurons. Since MPP^+ is a polar molecule, unlike its precursor MPTP, it cannot freely enter cells, but depends on the plasma membrane carriers to gain access to dopaminergic neurons. MPP^+ has a high affinity for plasma membrane dopamine transporter (DAT) [49], as well as for norepinephrine and serotonin transporters. The obligatory character of this step in the MPTP neurotoxic process is demonstrated by the fact that blockade of DAT by specific antagonists such as mazindol [32] or ablation of DAT gene in mutant mice [8] completely prevents MPTP-induced tox-

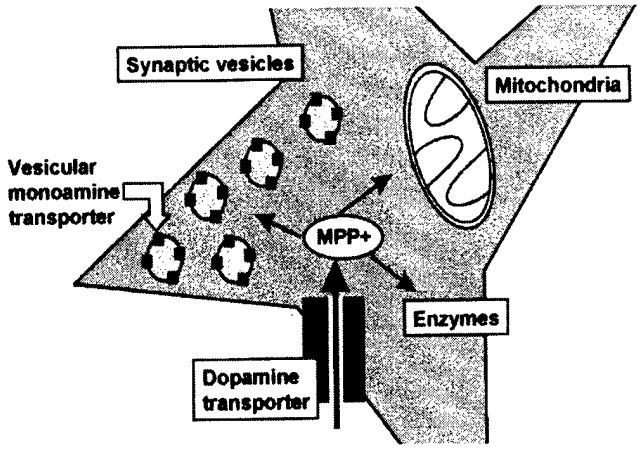

Fig. 2. Schematic representation of MPP^+ intracellular pathways. Inside dopaminergic neurons, MPP^+ can bind to the vesicular monoamine transporters (VMAT) and be translocated into synaptosomal vesicles, be concentrated by an active process within the mitochondria, and remain in the cytosol and interact with different cytosolic enzymes.

icity. Conversely, transgenic mice with increased brain DAT expression are more sensitive to MPTP [19].

Once inside dopaminergic neurons, MPP^+ can follow at least three routes as shown in Fig. 2: (i) it can bind to the vesicular monoamine transporters (VMAT) which will translocate MPP^+ into synaptosomal vesicles [45], (ii) it can be concentrated by an active process within the mitochondria [63], and (iii) it can remain in the cytosol and interact with different cytosolic enzymes [34]. The fraction of MPP^+ destined to each of these routes, is probably a function of MPP^+ intracellular concentration and affinity for VMAT, mitochondria carriers, and cytosolic enzymes. The importance of the vesicular sequestration of MPP^+ is demonstrated by the fact that cells transfected to express a greater density of VMAT are converted from MPP^+-sensitive to MPP^+-resistant cells [45]. Conversely, we demonstrated that mutant mice with 50 % lower VMAT expression are significantly more sensitive to MPTP-induced dopaminergic neurotoxicity compared to their wild-type littermates [71]. These findings indicate that there is a clear inverse relationship between the capacity of MPP^+ sequestration (i.e., VMAT density) and the magnitude of MPTP neurotoxicity.

Inside dopaminergic neurons, MPP^+ can also be concentrated by an active process within the mitochondria [63], where it impairs mitochondrial respiration by inhibiting complex I of the electron transport chain [50,55] through its binding at or near the same site as the mitochondrial poison rotenone [27,62]. The inhibition of complex I impedes the flow of electrons along the mitochondrial electron transport chain, leading to a deficit in ATP formation. It appears, however, that complex I activity should be reduced > 70 % to cause severe ATP depletion [15] and that, in contrast to *in vitro*, *in vivo* MPTP causes only a transient 20 % reduction in mouse striatal and midbrain ATP levels [12]. These findings raise the question as to whether MPP^+-related ATP deficit can be the sole factor underlying MPTP-induced dopaminergic neuronal death. Another consequence of complex I inhibition by MPP^+ is an increased production of free radicals, especially of superoxide [13,25,64]. From the abovementioned findings, it may be speculated that the initiation of MPP^+'s deleterious cascade of events may result from energy failure and oxidative stress, which individually may not be sufficient to kill cells, but in combination may well be lethal. A similar scenario of interplay among mitochondrial dysfunction, energy failure, and oxidative stress has been postulated for PD [4].

The importance of MPP^+-related superoxide production in dopaminergic toxicity process *in vivo* is demonstrated by the fact that transgenic mice with increased brain activity of copper/zinc superoxide dismutase (SOD1) are significantly more resistant to MPTP-induced dopaminergic toxicity than their non-transgenic littermates [61]. This finding strongly suggests that superoxide radical plays a pivotal role in the MPTP neurotoxic process. However, superoxide is poorly reactive, and it is the general consensus that this radical does not cause serious direct injury [23]. Instead, superoxide is believed to exert many or most of its toxic effects through the generation of other reactive species such as hydroxyl radical, whose oxidative properties can ultimately kill cells [23]. For instance, superoxide facilitates hydroxyl radical production by hydrogen peroxide and transitional metals such as iron (i.e., Fenton reaction, see Fig. 3) [23]. Although this reaction can readily take place *in vitro*, its occurrence *in vivo* is subordinate to such factors as low pH [44]. Despite this unfavorable pH constraint, MPTP does stimulate the formation of hydroxyl radicals *in vivo*, as evidenced by the increase in the hydroxyl radical-dependent conversion of salicylate into 2,3- and 2,5-dihydroxy-benzoates [66].

Superoxide can also react with NO to produce peroxynitrite (Fig. 3), another potent oxidant [6]. At physiological pH and in aqueous milieu, this reaction proceeds five times faster than the decomposition of superoxide by SOD [29]. The intracellular concentration of SOD1 is estimated at 10–40 μM [78]. Thus, NO concentration has to be ~10 μM for peroxynitrite

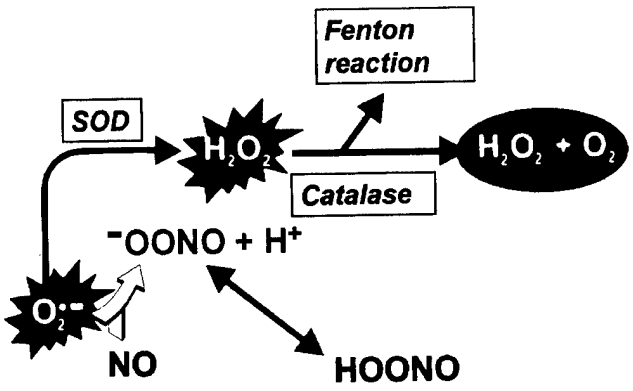

Fig. 3. Superoxide ($O_2^{\bullet-}$) can react with superoxide dismutase (SOD) to produce hydrogen peroxide (H_2O_2), which in turn, can react with catalase to produce water and oxygen or enter the Fenton reaction. Alternatively, superoxide can also react with nitric oxide (NO) to produce peroxynitrite ($^-$OONO) and peroxynitrous acid (HOONO).

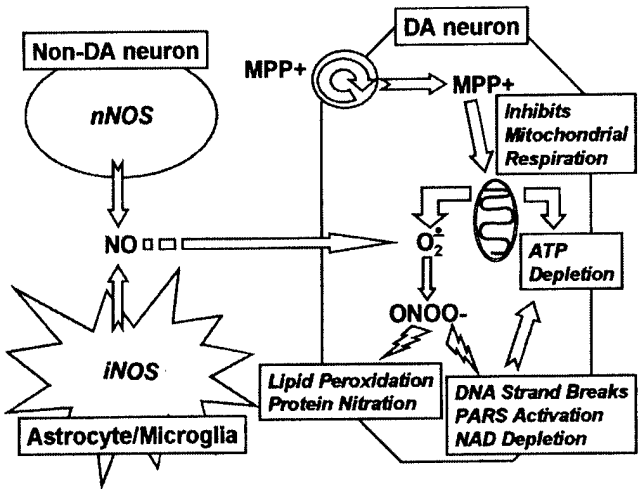

Fig. 4. Proposed scheme for selectivity of MPTP-induced dopaminergic neurotoxicity. MPP^+ is transported into dopaminergic neurons by the DAT. Then, MPP^+ inhibits enzymes in the mitochondrial electron transport chain, resulting in ATP deficit and increased "leakage" of superoxide ($O_2^{\bullet-}$) from the respiratory chain. Superoxide remains in the cell in which it is produced. On the other hand, NO, which is produced by nNOS and iNOS outside dopaminergic neurons, is membrane-permeable and can diffuse into neighboring neurons. If the neighboring cell has elevated levels of superoxide, then there is an increased probability of superoxide reacting with NO to form peroxynitrite, which can damage lipids, proteins, and DNA. Damaged DNA stimulates PARS activity, which further depletes ATP stores. In this scheme, it is the site of generation of superoxide which determines whether a cell will succumb to NO- and peroxynitrite-mediated deleterious effects. Since dopaminergic neurons selectively accumulate MPP^+, which in turn stimulates superoxide production, these neurons are selectively at risk.

formation to be competitive, which is not unrealistic as NO production at the cellular level is estimated at 1–10 μM [6]. The situation is different, however, for superoxide, whose basal intracellular concentration is low [7]. Thus, under normal conditions, superoxide is limiting, and it is likely that minimal peroxynitrite formation occurs (Fig. 3). Conversely, in pathological conditions, should superoxide concentrations increase, as in response to MPTP administration, formation of appreciable amounts of peroxynitrite is expected (Fig. 3). In light of this and of our previous work on superoxide [61], we [60] and others [24,66] have assessed the role of NO in the MPTP neurotoxic process. These studies show that inhibition of NO synthase (NOS) attenuates, in a dose-dependent fashion, MPTP-induced striatal dopaminergic loss in mice [60,66]. We also demonstrate that 7-nitroindazole (7-NI), a compound that inhibits NOS activity without significant cardiovascular effects in mice [51], is profoundly neuroprotective against MPTP-induced SNpc dopaminergic neuronal death [60]. The protective effect of the NOS antagonist 7-NI against MPTP-induced striatal and SNpc dopaminergic damage was subsequently demonstrated in monkeys [24].

4. MPTP proposed mechanism of action

From the above findings, the following scheme can be proposed to explain both selectivity and dopaminergic toxicity (Fig. 4): MPTP is converted to MPP^+ which is transported into dopaminergic neurons via the dopamine transporter. MPP^+ inhibits enzymes in the mitochondrial electron transport chain, resulting in ATP deficit and increased "leakage" of superoxide from the respiratory chain. Superoxide cannot readily transverse cellular membranes and so remains in the cell in which it is produced. In contrast, NO is membrane-permeable and diffuses into neighboring neurons. If the neighboring cell has elevated levels of superoxide, then there is an increased probability of superoxide reacting with NO to form peroxynitrite, which is highly reactive, damaging lipids, proteins, and DNA. In this scheme, it is the site of generation of superoxide which determines whether a cell will succumb to NO- and peroxynitrite-mediated deleterious effects. Since dopaminergic neurons selectively accumulate MPP^+, which in turn stimulates superoxide production, these neurons are selectively at risk.

5. Source of NO and NO synthase

As summarized above, there is strong evidence that NO participates in the MPTP neurotoxic process. Because MPTP selectively kills dopaminergic neurons, it is expected that the deleterious cascade of events that underlie the neurodegeneration takes place inside dopaminergic neurons. There are experimental arguments to indicate that superoxide concentration is, indeed, increased inside dopaminergic neurons by MPP^+. However, NOS which produces NO, has, thus far, not been identified inside dopaminergic neurons in rodents; although this needs to be confirmed, low levels of NOS might be present in dopaminergic neurons in humans [10]. In contrast to their lack of NOS, at least in rodents, dopaminergic structures are surrounded by NOS-containing fibers and cell bodies in the striatum, and, to a much lesser extent, in the SNpc [10,42]. Because NO is uncharged and lypophilic [38], it is able to travel away from its site of synthesis and inflict remote cellular damage without the need for any export mechanisms. It is suggested that NO, which is highly diffusible, can travel in random directions up to 150–300 mm during the 5–15 sec that correspond to its estimated half-life in physiological aqueous conditions [38]. Although this modeling may depart from the actual *in vivo* situation encountered by a molecule of NO, it gives credence to the hypothesis that NO can cover a distance several times greater than the diameter of a dopaminergic neuron. We are thus speculating that the NO production involved in MPTP toxicity takes place in non-dopaminergic cells present in the vicinity of dopaminergic structures.

Another question pertinent to the origin of NO in the MPTP model is which isoforms of NOS are primarily involved in this process? Nitric oxide is formed from arginine by NOS which oxidizes the guanidino nitrogen of arginine, releasing NO and citrulline. To date, three distinct NOS isoenzymes have been purified and molecularly cloned: neuronal NOS (nNOS, NOS I), inducible NOS (iNOS, NOS II), and endothelial NOS (eNOS, NOS III). Since all three iso-

forms of NOS have been identified in the brain, each of these can individually or in combination be involved in the production of NO used in MPTP neurotoxic process.

6. NOS isoforms

Neuronal NOS is the predominant isoform of NOS in the brain. Its catalytic activity and protein are identifiable throughout the brain [10,28]. Relevant to MPTP, nNOS is present in the striatum within intrinsic medium-sized neurons co-localizing somatostatin and neuropeptide Y [17]. In the midbrain, nNOS is found in cholinergic neurons and within serotoninergic fibers [17,42]. Thus, both by its abundance and its localization, nNOS appears to be an excellent candidate for producing NO for MPTP. In agreement with this is our demonstration that mutant mice deficient in nNOS are partially protected against MPTP-induced striatal dopaminergic toxicity [60]. The finding that mice are better protected by the NOS antagonist 7-NI than by the lack of nNOS expression suggests that although nNOS is important, it may not be the sole isoform of NOS that is involved in this neurotoxic process. Could it be iNOS?

In the normal brain, iNOS is not detectable [46] or is only minimally expressed [33]. However, under pathological conditions, iNOS expression can significantly increase in activated astrocytes as well as in other cells such as microglia [68] and invading macrophages. This was shown in the brain after kainic acid lesion [77], ischemic damage [54], and stab wound [68]. A similar scenario may exist in the MPTP model. Indeed, from our recent data, it appears, that early in the course of MPTP-induced dopaminergic neuron degeneration, there is an increase in midbrain iNOS activity within the strong astrocytic and microglial reactions that occur in the SNpc following MPTP administration. This recent study also shows that changes in iNOS activity are already substantial 24 hr after MPTP administration, which precedes the peak of dopaminergic neurodegeneration [31]. Therefore, NO derived from iNOS is likely minimal in normal brains, but may become increasingly substantial as MPTP-induced dopaminergic neurodegeneration progresses. Accordingly, iNOS may not play a significant role in the initiation of the MPTP toxic process, but may amplify it and assure its propagation by fueling dopaminergic neurons with increasing amounts of NO (Fig. 4).

7. Superoxide and nitric oxide

Superoxide is produced by many biological reactions, and especially by mitochondrial respiration [23]. It can be engaged in numerous reactions including the direct oxidation of biological molecules (e.g., cathechols) and the production of hydroxyl radicals. Similarly, NO exerts many biological effects that can be defined as direct (i.e., resulting from the reactions between NO and specific biological molecules) and indirect (i.e., resulting from the reactions between reactive nitric oxide species [RNOS], which are derived from NO oxidation, and specific biological targets) [78]. Most, if not all, of NO's direct effects appear to be related to biological regulatory effects, and not to neurotoxicity [78]. Although NO can directly affect mitochondrial respiration *in vitro* [9], the deleterious consequence of this effect remains to be determined *in vivo*. Conversely, NO's indirect actions, which are mediated by RNOS such as nitrite (NO_2^-), nitrate (NO_3^-), and peroxynitrite and its protonated derivative, peroxynitrous acid (N_2O_3H), are unquestionably deleterious [78]; in aqueous conditions, RNOS such as NO^+ and NO^- rapidly react with water and, thus, are unlikely to be major participants in noxious reactions.

8. Peroxynitrite and tyrosine nitration

In light of the above, it appears that, since they are weak oxidants, neither superoxide nor NO is, by itself, sufficiently damaging enough to participate directly in the MPTP toxic process. In contrast, we have also presented above different arguments supporting peroxynitrite in this role. The versatility of peroxynitrite as an oxidant is impressive [5,75]. For instance, an important aspect of peroxynitrite's deleterious action is the oxidation of phenolic rings in proteins, and in particular of tyrosine residues [57], to form nitrotyrosine as the most important product [76]. As such, detection and quantification of nitrotyrosine are important indirect evidence that peroxynitrite is involved in a pathological process. Relevant to the participation of peroxynitrite in the MPTP model, it has been demonstrated that MPTP significantly increases striatal levels of *free nitrotyrosine* in mice [66]. Although this finding provides major impetus to the implication of peroxynitrite in the MPTP model, one should be aware that the relationship between free and protein nitrotyrosine is unknown, and the physiopathologic role, if any, of free nitrotyrosine remains to be determined.

Aside from its role as a marker, nitrotyrosine can be a harmful modification as it can inactivate enzymes and receptors that depend on tyrosine residues for their activity [30,73] and prevent phosphorylation of tyrosine residues important for signal transduction [35,48]. This described cascade of events appears quite relevant to MPTP's mode of action as we have demonstrated that, following MPTP administration to mice, both striatal and midbrain levels of nitrotyrosine in proteins increase in a time-dependent fashion and that tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, becomes inactivated by tyrosine nitration [3].

9. DNA damage and poly(ADP-ribose) polymerase

Thus far, the lion's share of attention has been given to the effects of reactive species produced after MPTP administration on proteins. However, as stated above, most of the reactive species, like peroxynitrite, that may be implicated in the MPTP model, can damage, through oxidative processes, many vital cellular elements other than proteins [23].

Among these, DNA is of unique importance, because it is the repository for genetic information and is present in single copies. Oxidants like peroxynitrite can cause a range of DNA damage [23]. For example, DNA exposed to peroxynitrite produces 8-hydroxyguanine and 8-hydroxydeoxyguanosine; two modifications whose levels seem increased in midbrain of post-mortem PD brains compared to normal controls [2; 65]. Because peroxynitrite can nitrate the aromatic group, it can also stimulate the formation of 8-nitrodeoxyguanosine [11]. Finally, intact cells exposed to peroxynitrite exhibit a dose-dependent increase in DNA single strand breakage [70], which is another important type of DNA alteration. In light of the proposed oxidant species involved in MPTP neurotoxicity, all of these DNA modifications can possibly occur in this model, as well as in PD. However, despite the potential pathological role of DNA damage, to date we are not aware of any published study on this process in the MPTP model. Nevertheless, we have preliminary data generated in collaboration with Dr. M.F. Chesselet (Department of Neurology, UCLA) indicating that MPTP does indeed cause conspicuous DNA damage such as strand breaks in SNpc neurons of MPTP-treated mice.

Although all of the aforementioned modifications are potentially mutagenic and thus likely harmful, strand breakage is especially attractive because of its link to the enzyme poly(ADP-ribose) polymerase (PARP). Indeed, DNA single strand breakage is an obligatory trigger for the activation of PARP, a phenomenon that we believe, for the reasons that follow, to be a major factor in the overall MPTP-induced cascade of deleterious events. Thus far, the actual functions of PARP remain uncertain, and data obtained with cell-free systems and cells from PARP knockout mice suggest that, contrary to the common belief, PARP would not have a direct role in DNA repair mechanisms [43,69]. On the other hand, it is clear that the activation of PARP results in the cleavage of NAD^+ into ADP-ribose and nicotinamide, both *in vitro* and *in vivo* [43,69]. In turn, PARP covalently attaches ADP-ribose to diverse proteins, including nuclear proteins, histones, and PARP itself. PARP then extends the initial ADP-ribose groups into a nucleic acid group-like polymer, poly(ADP-ribose). It is, therefore, manifest that PARP activation, by synthesizing poly(ADP-ribose) polymer, can rapidly deplete intracellular stores of NAD^+ which may impair glycolysis and mitochondrial electron transport chain activities, and, consequently, ATP formation [43,69]. This PARP-dependent cascade of events could play a critical role in the demise of the SNpc dopaminergic neurons as suggested by *in vitro* and *in vivo* data [11,14,79]. This scenario may be even more significant if, as in the case of the MPTP model, the production of ATP in SNpc dopaminergic neurons is already compromised due to the inhibition of the mitochondrial complex I by MPP^+ [12] (Fig. 4). In favor of the importance of PARP activation in the MPTP neurotoxic process *in vivo* is our demonstration that mutant mice deficient in PARP are more resistant to MPTP-induced dopaminergic neuronal death [47].

10. Conclusions

The current understanding of the MPTP mode of action proposes that MPP^+ causes oxidative stress mediated by superoxide and NO. This leads to damage of proteins, lipids, and DNA, all contributing to major cellular dysfunctions. In addition, activation of reparative DNA enzymes, which consume ATP, aggravate the already reduced pool of ATP caused by the action of MPP^+ on complex I. This will impair numerous vital cellular reactions that are ATP-dependent. Subsequently, oxidative stress- and energy failure-related damage affect the cell's ability to maintain intracellular potential. Accordingly, intracellular potential will progressively rise until it reaches the threshold of activation of glutamate *N*-methyl-D-aspartate ionophor-channel and consequently triggers an excitotoxic insult [74]. This suggests that MPTP-induced cell death results from a complex interplay among mitochondrial dysfunction, oxidative stress, energy failure, and excitotoxicity. All of these key players will contribute to the impaired function of the cell until it becomes incompatible with life, and thus the cell dies by necrosis [31], if the injury is severe enough that no cellular function is preserved, or by apoptosis [72], if the injury is less severe and some cellular function is preserved. Because of the close similarity between PD and the MPTP model, it is likely that a similar cascade of deleterious events underlies the pathogenesis of PD.

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Experimental developments in movement disorders: update on proposed free radical mechanisms

Serge Przedborski and Vernice Jackson-Lewis

Free radicals have been implicated in the pathogenesis of movement disorders such as Parkinson's disease and Huntington's disease. Some basic aspects about free radicals as they relate to oxidative stress in neurodegeneration are summarized. Old and new experimental findings pertinent to oxidative damage in movement disorders are reviewed. Finally, the degree to which toxin-induced and genetically engineered experimental models have been useful in delineating parts of the mechanisms involved in the cascade of events that lead to neuronal death is emphasized. Curr Opin Neurol 11:335–339.

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Abbreviations

MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
3-NP	3-nitropropionic acid
SOD	superoxide dismutase

Introduction

Movement disorders are neurologic conditions that are characterized by increased abnormal movements or, on the contrary, paucity of movements. The former are typically referred to as hyperkinetic movement disorders and they are exemplified by Huntington's disease, whereas the latter are called hypokinetic movement disorders and are exemplified by Parkinson's disease. Most but not all of these neurologic disorders are associated with dramatic premature degeneration of specific subsets of neurons in the basal ganglia or the substantia nigra, variable degrees of gliosis, and often intracellular inclusions of protein nature.

Over several years, major progress has been achieved in our understanding of the etiologies of these diseases. One of the most striking examples of this is the discovery that the etiology of Huntington's disease is related to an expansion of CAG (polyglutamine) trinucleotide repeats in the encoding region of its gene. However, we still have little indication as to what is the pathogenesis of these disorders, including Huntington's disease. Indeed, although we know the etiology of Huntington's disease, we do not know how the produced abnormal protein with expanded CAG tract leads to neurodegeneration. We acknowledge that although we need to elucidate the etiologies of these diseases, it is as important to search for their pathogeneses, because our ultimate goal, which is to cure these disorders, may require an understanding of both as the research trail of Huntington's disease illustrates so well. Among different proposed pathogenic mechanisms, a large number of investigators including ourselves have been particularly attracted by the potential role of free radicals as causative or contributing factors in the pathogenesis of neurodegenerative, and more specifically, movement disorders. Although a free-radical-mediated mechanism has been speculated for several movement disorders, it is important to mention that the model of oxidative damage *par excellence* remains Parkinson's disease [1], and to a lesser extent Huntington's disease [2].

Free radicals and the brain

Before reviewing the literature about movement disorders and oxidative stress, it is important to summarize some of the basic aspects related to the biology of free radicals, cellular mechanisms of defense, and why the brain is a particularly attractive target for free radical attack. This summary focuses strictly on those aspects pertinent to neurodegeneration and movement disorders, and does not

review the question in an exhaustive manner. For an in-depth review of the subject, refer to [3,4].

Electrons within atoms and molecules exist in pairs and occupy space known as an orbital. When an orbital contains only one electron, that electron is said to be unpaired, and the corresponding atom or molecule is said to be a free radical. Because this electron imbalance renders the free radical unstable, it tends to acquire greater stability by completing its orbital through the removal of an electron from neighboring atoms or molecules. As a result, the latter have altered electron organization, which can have dramatic structural or functional implications; this is called oxidative damage. Free radicals can cause oxidative damage to virtually all cellular components, including DNA, proteins, and membrane lipids. It can, thus, be hypothesized that in neurodegenerative disorders such as Parkinson's disease, there is a sustained oxidative stress that causes a progressive build up of cellular alterations, which can ultimately lead to cell death.

Free radicals are produced constantly during normal cellular metabolism. However, defense mechanisms exist to limit the levels of free radicals and the damage they inflict on cells. These include scavenging enzymes, such as superoxide dismutase (SOD), the main function of which is to detoxify the superoxide radical, and glutathione peroxidase and catalase, the main function of which is to scavenge hydrogen peroxide. There are also several small molecules that exhibit antioxidant properties and play an important role in preventing oxidative damage, such as the reduced form of glutathione, β-carotene, ubiquinol, α-tocopherol (vitamin E), and ascorbic acid (vitamin C). It has been hypothesized that the fine-tuned balance between the production and the destruction of free radicals is upset in pathologic conditions, such as Parkinson's disease and Huntington's disease, resulting in an overall increase in the levels of free radicals.

Although a similar pathologic scenario may apply to any system/organ in our body, the brain has received the majority of attention, mainly because it is recognized as particularly susceptible to oxidative stress: it has a very high oxygen consumption rate relative to its weight; it contains a relatively low arsenal of free radical defense mechanisms; and it contains a remarkably high concentration of unsaturated lipids, which are 'easy targets' for free radical attack.

Does oxidative stress contribute to neurodegeneration?

After reading the above summary aimed at laying the groundwork for the oxidative stress hypothesis in neurologic diseases, one may wonder whether there is actually experimental evidence to support the view that free radicals are implicated in the pathogenesis of movement disorders.

The first possibility concerns whether the postulated oxidative stress in movement disorders is due to insufficient protection. The initial line of defense against free radicals is SOD. This abundant enzyme comes in three forms: two copper/zinc SODs (cytosolic SOD and extracellular SOD) and a manganese SOD (mitochondrial SOD). Paradoxically, manganese SOD protein expression [5] and activity [6] is increased in Parkinson's disease; copper/zinc SOD is unchanged [7]. Other abnormalities in the free radical protective mechanisms include reduced concentrations in the reduced form of glutathione [8], mild alteration in catalase [9], and minimal to no changes in glutathione peroxidase [10,11]. In addition, low ubiquinone levels were found in platelets of Parkinson's disease patients [12]. All of these, with the exception of manganese SOD, show changes that are consistent with the view that in Parkinson's disease a situation of oxidative damage due to an insufficient protection cannot be excluded.

The second possibility, which is not mutually exclusive, is the postulated oxidative stress in movement disorders due to increased production of free radicals. Several markers of oxidative damage have been reported to be increased in the substantia nigra of Parkinson's disease patients. For instance, concentrations of malonaldehyde [13] and of 4-hydroxynoneal protein adducts [14], two markers of lipid peroxidation, are significantly increased in the substantia nigra of Parkinson's disease patients. Increased levels of 8-hydroxydeoxyguanosine, a marker of oxidative damage to DNA, has also been reported in Parkinson's disease [15,16]. Proteins do not escape attack because carbonyl content, a free-radical-mediated modification of proteins, is similarly increased in Parkinson's disease autopsy brain samples [17]. Some, but not all of these alterations were also identified in Huntington's disease brains [18•]. Collectively, these data support the hypothesis that in Parkinson's disease and, possibly in Huntington's disease, neurons are the site of an oxidative stress. However, none of them demonstrate that there is an actual increase in the formation of free radicals. To date, the only realistic sources for an increase in the formation of free radicals would be the mitochondrial electron transport chain and selected intracellular enzymes, such as those involved in dopamine metabolism. The main source of intracellular free radicals is the mitochondrial electron transport chain. Several enzymatic complexes of the electron transport chain are impaired in Parkinson's disease [19] and Huntington's disease [20,21]. Thus, the mitochondrial defects observed in Parkinson's disease and Huntington's disease by impairing electron flow may not only lead to a deficit in ATP production, but also to an increase in free radical formation. This speculation suggests that the deleterious cascade of events that leads to neurodegeneration in Parkinson's disease and Huntington's disease may be an interplay between mitochondrial dysfunction, energy failure, and oxidative stress. Among

the different enzymes implicated in dopamine metabolism, it has long been known that monoamine oxidase produces different reactive oxygen species during the catabolism of dopamine [1]. Recent, however, is the demonstration that tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis, can also stimulate the production of free radicals [22•]. During the neurodegenerative process in Parkinson's disease, the spared neurons are the sites of increased dopamine turnover [23]. This adaptive mechanism, although with good intention, increases the load of free radicals per spared neuron and may contribute to the progression of the disease.

A caveat, however, with most if not all of these studies is the fact that they are autopsy studies; thus, they deal with end-stage diseased brains, which are dramatically depleted of the targeted neurons and are 'contaminated' by adaptive responses. Also, this does not even consider another confounding factor, which is the chronic administration of drugs such as L-dihydroxyphenylalanine, which can trigger its own oxidative stress [24,25].

Free radicals and toxin-induced neurodegeneration

In addition to the above arguments, another limitation in the use of autopsy samples in the exploration of the pathogenesis of diseases resides in the fact that they provide a 'snapshot' and not a 'dynamic system', which, no doubt, would be much better suited to the search for deleterious mechanisms. Accordingly, the past few years have witnessed an explosion of in-vitro and in-vivo model systems using neurotoxins.

Among the large variety of neurotoxins that do exist, by far the most popular toxin, which has provided the greatest insights into Parkinson's disease, is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [26]. A recent neurotoxin, already with major contributions, is 3-nitropropionic acid (3-NP), a neurotoxin that models Huntington's disease [27]. Indisputably, these two toxins, which have been extensively studied in various animal species, have provided important hints into the pathogenesis of Parkinson's disease and Huntington's disease, respectively. Both compounds replicate behavioral abnormalities in nonhuman primates, which are similar to those seen in their respective human disease conditions [28,29]. They also affect the mitochondrial electron transport chain and stimulate free radical production. For instance, transgenic mice with increased copper/zinc SOD activity are resistant both to MPTP [30] and to 3-NP [31]. Moreover, knock-out mice deficient in neuronal nitric oxide synthase, the enzyme that produces nitric oxide, are also resistant to MPTP [32] and to malonate, a toxin that, like 3-NP, inhibits the same mitochondrial enzyme [33]. These studies suggest that both superoxide and nitric oxide are implicated in MPTP toxicity, and possibly in 3-NP/malonate-mediated toxic process. However, superoxide and nitric oxide are poorly reactive, and it is the general consensus that these radicals do not cause serious direct injury. Instead, superoxide and nitric oxide are believed to exert many or most of their toxic effects through the generation of other reactive species, such as hydroxyl radical, the oxidative properties of which can ultimately kill cells. Indeed, superoxide can react with iron to produce hydroxyl radicals through the transition metal-catalyzed Fenton reaction. The production of hydroxyl radical by MPTP is supported by the demonstration of a significant increase in the formation of 2,5-dihydroxybenzoic acid, the reaction product of salicylate and hydroxyl radical in MPTP-treated mice [34,35]. The importance of iron in the MPTP toxic process is illustrated by fact that in rat brain dialysis experiments desferrioxamine, an iron chelator, protects against 1-methyl-4-phenylpyridinium, the active metabolite of MPTP [36,37]. Superoxide can also combine with nitric oxide to produce peroxynitrite, a extremely reactive species with versatile damaging properties [38]. The increased formation of peroxynitrite after MPTP administration is demonstrated by a rise in the levels of nitrotyrosine [35], a permanent protein modification caused by peroxynitrite [39]. Not to be ignored is the neurotoxin *N*-methyl-(R)-salsolinol, which, in contrast to MPTP, has been identified in Parkinson's disease brains and causes behavioral changes in rats that are reminiscent of Parkinson's disease [40]. This potential endogenous parkinsonian toxin selectively affects substantia nigra dopaminergic neurons [40] and stimulates the production of free radical in in-vitro systems [41].

Free radicals and genetic models

Overexpression of free radical scavenging enzymes in transgenic mice has provided tools to examine the effects of increased protection of the brain against different types of insults. As mentioned above, transgenic mice with increased copper/zinc SOD activity provide protection against neurotoxins. In contrast, overexpression of glutathione peroxidase in PC-12 cells protects against methamphetamine, but not against MPTP [42•]. Of note is the fact that mutations in copper/zinc SOD cause motor neuron death replicating the hallmarks of amyotrophic lateral sclerosis [43]. Although the molecular mechanism of mutant copper/zinc SOD remains unclear, there is evidence pointing to the fact that mutant copper/zinc SOD stimulates oxidative stress [43]. In light of this, it is pertinent to mention that not only does mutant copper/zinc SOD kill motor neurons of the spinal cord, but it also destroys substantia nigra dopaminergic neurons [44], supporting the vulnerability of dopaminergic cells to oxidative stress. Reverse experiments, nullifying free radical scavenging enzymes in knock-out mice, have been even more illuminating. The ablation of copper/zinc SOD and extracellular SOD have minimal effects on the

development and life span of the mutant animals and they do not exhibit abnormal movements [45,46]. Conversely, the effects of ablating manganese SOD were both immediate and dramatic [47,48]. Even though the mice were healthy at birth, they grew poorly and developed sustained muscle contractions resembling dystonia, as well as head tremor by the age of 2 weeks [48,49•]. Interestingly, these mice did not exhibit any evidence that oxidative stress extended beyond the mitochondria; for example, there was no increase in lipid peroxidation. Free radical attack in the manganese SOD knock-out mice may well be restricted to mitochondria. The combination of a short life span and the development of a movement disorder, in the context of free-radical-mediated damage to mitochondria, favors the hypothesis that the premature death of selected subsets of neurons in movement disorders may be related to similar mitochondrial damage.

Conclusion

This review has summarized some of the literature concerning recent developments in free radical research with regard to movement disorders. As we can see, there is a tremendous amount of ongoing work in favor of the free radical hypothesis in the pathogenesis of movement disorders. It is remarkable that many of the same abnormalities, such as tyrosine nitration, lipid peroxidation and mitochondrial dysfunction, are common to a number of movement disorders. This raises the possibility, as we speculate, that neurodegenerative disorders may differ in their phenotypic expression and etiologic factors, but may share a similar cascade of deleterious events. Alternatively, it is also possible that most of the identified abnormalities are secondary to neurodegeneration and can be seen each time a cell is committed to die, regardless of the specific mechanism of demise. However, it is still debatable whether the free radical is the principle culprit in the death of neurons in movement disorders. We can now hope to build a stronger case for the oxidative hypothesis through intensive research aimed at developing more experimental models that mimic human disease states (e.g. transgenic Huntington's disease mice) or that replicate putative pathogenic mechanisms (e.g. knock-out manganese SOD mice).

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Mechanisms of MPTP Toxicity

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Summary: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) produces an experimental model of Parkinson's disease (PD). It replicates most of the clinical features of PD as well as the main biochemical and pathologic hallmarks of the disease. Although the MPTP model departs from PD in several aspects, it is thought that important insights into the neurodegenerative process of PD may be obtained by elucidating the molecular mechanism of MPTP. In this article, we summarize the different steps of the complex metabolic pathway of MPTP and show how they may be implicated in predisposing individuals to PD. We also outline findings pertinent to the mode of action of MPTP including overproduction of free radicals, implication of nitric oxide, nitration of tyrosine, impairment of mitochondrial respiration, and occurrence of apoptosis. All of these factors may participate in the cascade of deleterious events that ultimately lead to the death of dopaminergic neurons after MPTP administration. Because of the similarity between PD and the MPTP model, we are speculating that a similar scenario may underlie the neurodegenerative process in PD. **Key Words:** Parkinson's disease—MPTP—Pathogenesis—Free radicals—Mitochondria.

Parkinson's disease (PD) is a common neurodegenerative disorder characterized mainly by resting tremor, slowness of movement, rigidity, and postural instability which is associated with a dramatic loss of dopamine (DA) neurons in the substantia nigra pars compacta (SNpc).[1] The prevalence of PD has been estimated at ~1,000,000 in North America with ~50,000 newly affected individuals each year.[1] Thus far, the most potent treatment for PD remains the administration of L-dopa, a precursor of DA, which replenishes brain DA, thus alleviating almost all PD symptoms.[1] However, the chronic administration of L-dopa often causes motor and psychiatric side effects, which may be as debilitating as PD itself.[2] Furthermore, there is no evidence to support the fact that L-dopa therapy impedes the progressive death of SNpc DA neurons. This leads PD patients, in a few years after the beginning of the symptoms, to a painful dilemma: take no or low doses of L-dopa to avoid the side effects and be severely parkinsonian, or take high doses of L-dopa to control PD symptoms and be subjected to severe side effects. Therefore, without undermining the importance of L-dopa therapy in PD, there is an urgent need to acquire a deeper understanding of the cause of PD, not only to prevent the disease, but also to develop therapeutic strategies aimed at halting its progression in newly diagnosed patients whose minimal disability does not require L-dopa administration. To this end and in light of the rarity of available postmortem brain samples from PD patients for neuropathologic studies, many investigators, including ourselves, have focused their research efforts on experimental models of PD such as the one produced by the toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).

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MPTP—A MODEL OF PARKINSON'S DISEASE

The fact that MPTP causes a parkinsonian syndrome was discovered in 1982 when a group of drug addicts in California were rushed to an emergency room with a severe bradykinetic and rigid syndrome.[3] Subsequently, it was discovered that this syndrome was induced by the self-administration of street batches of a synthetic heroin analogue whose synthesis had been heavily contaminated by a byproduct, MPTP.[3] In a period of a few days after the administration of MPTP, these patients exhibited a severe and irreversible akinetic–rigid syndrome. The analogy to PD was rapidly made by Dr. Langston and his group, and L-dopa was tried with success in relieving these unfortunate patients' symptoms.

Subsequently, MPTP has been used extensively and successfully in various mammalian species, including monkeys and mice, to produce an experimental model of PD.

The similarity between PD and the MPTP model is striking. All clinical signs of PD were recognized in humans and monkeys intoxicated with MPTP. This goes beyond the association of tremor, rigidity, akinesia, and postural instability, because masked face, increased seborrhea, and freezing have been described in both humans and monkeys. MPTP-intoxicated humans and monkeys responded to L-dopa similarly to PD patients, including the development of side effects seen after its chronic use. Even transplantation surgery of fetal dopaminergic grafts dramatically improved the condition of MPTP-intoxicated individuals better than the response from PD patients to a similar approach. As in PD, the main target of MPTP is the SNpc DA neurons that, similar to PD, are significantly more damaged than those of the ventral tegmental area (dopaminergic structure directly medial to the SNpc). In monkeys injected with low doses of MPTP, it was also demonstrated that the loss of DA nerve terminals predominated in the putamen over the caudate, which is also a typical neuropathologic picture seen in PD. Also in monkeys, intraneuronal inclusions resembling Lewy bodies, which are typical features of the neuropathology of PD, were seen in aged MPTP-treated monkeys. In both PD and MPTP models it is believed that free radical toxicity (also called oxidative stress hypothesis) plays a determinant role in the overall cascade of deleterious events leading to the death of the dopaminergic neurons. Activity of complex I of the mitochondrial electron transport chain has been reported to be deficient in PD brains, and is exactly the same complex of the electron transport chain that is blocked by MPTP's active metabolite.

For these reasons, it is widely accepted that the MPTP model of PD is by far the best available experimental model for this disease. The MPTP model, however, fails to replicate some other aspects of PD. For example, PD is a slowly progressive disorder whose neurodegenerative process extends over several years. In contrast, in MPTP in humans, monkeys, and mice, the end point of the damage is reached after only a few days. Lewy bodies, which are regarded as a key feature of PD, have not yet been unequivocally demonstrated in MPTP models. Furthermore, MPTP or any related compounds have never been recovered from PD patients' brains or body fluids. Although these discrepancies strongly support the contention that PD is not the result of MPTP, the large number of similarities between the PD and the MPTP model support the commonly accepted view that significant insights into the molecular mechanisms of PD can be achieved through the study of the MPTP model.

MODE OF ACTION OF MPTP

MPTP has a complex multistep metabolism.[4] It is a highly lipophilic compound. Thus, after its systemic administration, it can quickly gain access to the brain by freely crossing the blood–brain barrier. Once in the brain, MPTP, which is a pro-toxin, is converted into its active metabolite, 1-methyl-4-phenylpyridinium (MPP+), by monoamine oxidase type B within non-dopaminergic neurons (for example, serotonergic neurons and glial cells). The next important step in the MPTP neurotoxic pathway is the mandatory entry of MPP+ into DA neurons. Because MPP+ is a polar molecule, contrary to its precursor MPTP, it can not freely enter into cells, but depends on the plasma membrane carriers. MPP+ has a high affinity for plasma membrane DA transporter (DAT) as well as for norepinephrine and serotonin transporters. The obligatory character of this step in the MPTP neurotoxic process is demonstrated by the fact that blockade of DAT by specific antagonists (for example, mazindol) completely prevents MPTP-induced toxicity. If decreasing DAT availability reduces MPTP toxicity, one may wonder whether increasing DAT availability enhances it. To address this question, we studied MPTP neurotoxicity in transgenic mice with increased brain DAT expression. These transgenic mice have ~25% higher density in DAT in the SNpc. We found that MPTP administration reduced the number of dopaminergic neurons both in the wild-type and the transgenic animals.[5] However, the reduction was significantly greater in the transgenic animals with higher DAT expression.[5] This observation suggests that individuals with higher DAT density may be at risk of developing PD should the disease be the result of an endogenous or exogenous toxin entering dopaminergic neurons through DAT. Relevant to this point is the demonstration that 15–25% variation in DAT density exists among humans and nonhuman primates.[6]

Inside the DA neurons, MPP+ can be taken up by the vesicular monoamine transporters (VMATs) and sequestrated within synaptosomal vesicles. The importance of this vesicular sequestration of MPP+ is demonstrated by the fact that cells transfected to express greater density of VMAT are converted from MPP+-sensitive to MPP+-resistant cells.[7] Conversely, we demonstrated that mutant mice with 50% lower VMAT expression are significantly more sensitive to MPTP-induced DA neurotoxicity compared with their wild-type littermates.[8] Altogether, these findings indicate that there is a clear inverse relationship between the capacity of MPP+ sequestration

(that is, VMAT density) and the magnitude of MPTP neurotoxicity. This raises the question of whether VMAT antagonists (for example, reserpine, tetrabenazine) may not increase the risk of developing PD or exacerbate PD neurodegeneration.

Intracellular MPP+ can also be taken up and concentrated within the mitochondria where it blocks complex I of the electron transport chain, which in turn decreases the production of ATP and increases the formation of free radicals such as superoxide. Although the actual impact of MPP+-induced ATP deficit in MPTP neurotoxicity remains to be demonstrated in living animals, we have supported the critical role played by free radical production in the MPTP toxic process. This issue was assessed by testing MPTP toxicity in transgenic mice with increased activity of superoxide dismutase (SOD), the key enzyme in the detoxification of superoxide. These mice have 2.5–3 times greater brain SOD activity compared with wild-type littermates.[9] We found that MPTP administration caused significant damage in the wild-type animals, but in contrast, no significant damage was detected in the transgenic animals with increased SOD.[10] These data enabled us to conclude that superoxide plays a role in the molecular mechanism of MPTP in vivo. However, superoxide is probably better known as being an intermediate species in various cellular reactions rather than being deleterious by itself. In contrast, superoxide can react with nitric oxide (NO) to produce highly reactive and deleterious species such as peroxynitrite. Consistent with the idea that part of the MPTP neurotoxic process involves the reaction between superoxide and NO is the demonstration by us[11] and others[12,13] that inhibition of nitric oxide synthetase (NOS), the enzyme that synthesizes NO, protects, in a dose-dependent fashion, against MPTP.

In an attempt to examine the role of mitochondrial electron transport chain blockade in the MPTP neurotoxic process, we assessed MPTP toxicity in Rho 0 cells, which are mutant cells completely lacking electron transport chain activity.[14] We were fascinated by the observation that MPP+ toxicity in the Rho 0 cells was dose-dependent comparable to that found in their parental cells, which have normal mitochondrial function.[15] We were also able to demonstrate that not only was the toxicity comparable in these two lines, but that the proportion of death occurring by a morphology of necrosis or apoptosis was also comparable between Rho 0 cells and parental cells.[15] Although Rho 0 cells represent a peculiar system and caution should be taken in interpreting this finding, it suggests that MPP+ may kill cells by mechanisms that are not solely related to mitochondrial defect.

CONCLUSION

The current understanding of the MPTP mode of action proposes that MPP+ causes oxidative stress mediated by superoxide and NO. This leads to damage of proteins, lipids, and DNA, all contributing to major cellular dysfunctions. In addition, activation of reparative DNA enzymes, which consume ATP, aggravate the already reduced pool of ATP caused by the action of MPP+ on complex I. This will impair numerous vital cellular reactions that are ATP-dependent. Subsequently, oxidative stress and energy failure-related damage affect the cell's ability to maintain intracellular potential. Accordingly, intracellular potential will progressively rise until it reaches the threshold of activation of glutamate N-methyl-D-aspartate ionophor-channel and consequently triggers an excitotoxic insult.[16] This suggests that MPTP-induced cell death results from a complex interplay among mitochondrial dysfunction, oxidative stress, energy failure, and excitotoxicity. A similar scenario has been proposed for PD.[17] All of these key players will contribute to the impaired function of the cell until it becomes incompatible with life, and thus the cell dies by necrosis[18] if the injury is so severe that no cellular function is preserved, or by apoptosis[19] if the injury is less severe and some cellular function is preserved.

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Mass Spectrometric Quantification of 3-Nitrotyrosine, *ortho*-Tyrosine, and *o,o'*-Dityrosine in Brain Tissue of 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated Mice, a Model of Oxidative Stress in Parkinson's Disease*

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Oxidative stress is implicated in the death of dopaminergic neurons in Parkinson's disease and in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinson's disease. Oxidative species that might mediate this damage include hydroxyl radical, tyrosyl radical, or reactive nitrogen species such as peroxynitrite. In mice, we showed that MPTP markedly increased levels of *o,o'*-dityrosine and 3-nitrotyrosine in the striatum and midbrain but not in brain regions resistant to MPTP. These two stable compounds indicate that tyrosyl radical and reactive nitrogen species have attacked tyrosine residues. In contrast, MPTP failed to alter levels of *ortho*-tyrosine in any brain region we studied. This marker accumulates when hydroxyl radical oxidizes protein-bound phenylalanine residues. We also showed that treating whole-brain proteins with hydroxyl radical markedly increased levels of *ortho*-tyrosine *in vitro*. Under identical conditions, tyrosyl radical, produced by the heme protein myeloperoxidase, selectively increased levels of *o,o'*-dityrosine, whereas peroxynitrite increased levels of 3-nitrotyrosine and, to a lesser extent, of *ortho*-tyrosine. These *in vivo* and *in vitro* findings implicate reactive nitrogen species and tyrosyl radical in MPTP neurotoxicity but argue against a deleterious role for hydroxyl radical in this model. They also show that reactive nitrogen species and tyrosyl radical (and consequently protein oxidation) represent an early and previously unidentified biochemical event in MPTP-induced brain injury. This finding may be significant for understanding the pathogenesis of Parkinson's disease and developing neuroprotective therapies.

Parkinson's disease (PD)[1] is attributed to a profound deficit in dopamine (1) that follows the loss of dopaminergic neurons in the substantia nigra pars compacta and dopaminergic nerve terminals in the striatum (2). Although the mechanisms are uncertain, a large body of experimental evidence implicates oxidative stress (reviewed in Refs. 3–5). In light of these human and animal studies, at least three pathways have been proposed.

Studies with the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD suggest a pivotal role for superoxide radical ($O_2^{\cdot-}$) both *in vitro* and *in vivo* (6–10). $O_2^{\cdot-}$ is not highly reactive, however, and is generally thought not to cause serious direct injury (4, 11). Instead, it is believed to exert many or most of its toxic effects by generating reactive species such as hydroxyl radical ($HO^{\cdot}$), whose oxidative properties can ultimately kill cells (4, 11). For instance, $O_2^{\cdot-}$ facilitates $HO^{\cdot}$ production in the metal-catalyzed Haber-Weiss reaction both by reducing redox-active transition metals (M^{n+}) and by dismutating to form hydrogen peroxide (H_2O_2) (4, 11).

$$M^{n+} + O_2^{\cdot-} \rightarrow M^{n+-1} + O_2$$
$$M^{n+-1} + H_2O_2 \rightarrow M^{n+} + HO^{\cdot} + HO^-$$

REACTIONS 1 AND 2

This noxious reaction may be relevant to PD because the postmortem concentration of nonheme iron is dramatically elevated in the substantia nigra pars compacta of PD patients (12, 13). Moreover, neuromelanin, believed to be a by-product of dopamine autooxidation, may promote the formation of reactive species such as $HO^{\cdot}$ through various mechanisms (14).

Superoxide can also react with nitric oxide (NO) to produce peroxynitrite ($ONOO^-$), another potent oxidant (15).

$$O_2^{\cdot-} + NO \rightarrow ONOO^-$$

REACTION 3

Under normal conditions, $O_2^{\cdot-}$ may be limiting, resulting in little $ONOO^-$ formation. Appreciable amounts could form if $O_2^{\cdot-}$ concentrations increased, as in the response to MPTP or PD, however. NO is implicated by the observation that nitric-oxide synthase inhibitors attenuate MPTP-induced dopaminergic

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[1] The abbreviations used are: PD, Parkinson's disease; BHT, butylated hydroxytoluene; DTPA, diethylenetriaminepentaacetic acid; GC/MS, gas chromatography-mass spectrometry; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; HPLC, high performance liquid chromatography.

neurotoxicity in mice and monkeys (16–18). Mutant mice deficient in neuronal nitric-oxide synthase also are resistant to MPTP (16).

While investigating oxidative damage in atherosclerosis and other inflammatory conditions (5, 19), we described an oxidative pathway that does not require free metal ions. It involves myeloperoxidase, a heme protein secreted by activated phagocytes (20). Myeloperoxidase uses H_2O_2 to convert the phenolic amino acid tyrosine into a reactive intermediate that promotes the oxidation of proteins and lipids (21–23). We have recently used electron paramagnetic resonance spectroscopy to demonstrate that the oxidizing intermediate generated by myeloperoxidase and other peroxidases is free tyrosyl radical (24). These studies support the idea that tyrosyl radical may promote oxidative reactions at sites of inflammation.

One powerful strategy for understanding the underlying mechanisms of oxidative injury is to identify stable end products of protein oxidation by different reaction pathways *in vitro* and then to determine whether these markers are present at elevated levels *in vivo* (19, 25). For instance, HO$^{\cdot}$ converts protein-bound phenylalanine residues to the unnatural amino acid isomer *ortho*-tyrosine. Tyrosyl radical forms *o,o'*-dityrosine as the major product, whereas $ONOO^-$ generates 3-nitrotyrosine. These compounds are stable to acid hydrolysis, making them potentially useful markers of protein oxidation *in vivo*.

Immunohistochemical and HPLC methods have been used to detect products such as 3-nitrotyrosine (26, 27), but both approaches may be confounded by structurally distinct molecules. Moreover, antibody-based analyses are semi-quantitative at best. HPLC can be more sensitive and specific than immunohistochemistry, but brain tissue may contain materials that interfere with this method (27). Therefore, the hypothesis that neuronally derived NO reacts with O_2^- to generate $ONOO^-$ remains questionable, as does the idea that HO$^{\cdot}$ is produced in the MPTP model and PD.

Using isotope dilution gas chromatography-mass spectrometry (GC/MS), we developed sensitive and highly specific quantitative assays for measuring tissue levels of *ortho*-tyrosine, *o,o'*-dityrosine and 3-nitrotyrosine (25). We then analyzed the relative product yields of each marker in brain proteins oxidized *in vitro* by HO$^{\cdot}$, tyrosyl radical, or $ONOO^-$. We also assayed the oxidized amino acids in brain proteins from control and MPTP-treated mice. Our results suggest that reactive nitrogen species and tyrosyl radical, perhaps generated by $ONOO^-$ or heme proteins, mediate protein oxidation in this mouse model of PD.

EXPERIMENTAL PROCEDURES

Materials—Reagents were obtained from either Sigma or Aldrich unless otherwise specified. All organic solvents were HPLC grade. Cambridge Isotope Laboratories (Andover, MA) supplied ^{13}C-labeled amino acids for the preparation of internal standards. *o,o'*-[$^{13}C_{12}$]Dityrosine and *ortho*-[$^{13}C_6$]tyrosine were synthesized as described previously (21). 3-Nitro[$^{13}C_6$]tyrosine was synthesized using tetranitromethane (28). Concentrations of ^{13}C-labeled amino acids were determined by HPLC analysis (29).

Animals—Male C57/BL mice (8 weeks old; 22–25 g; Charles River Breeding Laboratories) were used in the study. Animals were housed three per cage in a temperature- and light-controlled room with a 12-h/12-h light-dark cycle. The mice were provided with water and food *ad libitum*. On the day of the experiment, mice received four intraperitoneal injections of MPTP-HCl (20 mg/kg; Research Biochemicals, Natick, MA) in saline every 2 h over an 8-h period. Control mice received saline only. All procedures followed National Institutes of Health guidelines for the use of live animals and were approved by the Columbia University Institutional Animal Care and Use Committee.

Collection of Tissues—Animals were anesthetized and sacrificed 24 h after the last injection; this time point was based on our previous study of nitration of tyrosine hydroxylase in the MPTP mouse model (30). To minimize *ex vivo* oxidation, mice were perfused with ice-cold antioxidant buffer (100 μM diethylenetriaminepentaacetic acid (DTPA; a metal chelator), 1 mM butylated hydroxytoluene (BHT; an inhibitor of lipid peroxidation), 10 mM 3-amino-1,2,4-triazole (an inhibitor of peroxidases and nitric-oxide synthase), 50 mM sodium phosphate, pH 7.4). Then, cerebellum, ventral midbrain, striatum, and cerebral cortex were dissected out on an ice-cold plate, frozen on dry ice, and stored at −80 °C until analysis (30).

Isolation of Brain Proteins for in Vitro Oxidation Studies—Freshly prepared sample from the indicated brain area was homogenized at 4 °C in 5 ml of buffer A (0.1 mM DTPA, pH 7.4), freeze-thawed once, and centrifuged at 10,000 × *g* for 10 min. The low speed supernatant was dialyzed against distilled, deionized water that had been passed over a Chelex resin (Bio-Rad) column to remove free metal ions.

Protein Oxidation by Hydroxyl Radical, Myeloperoxidase, and Peroxynitrite—In vitro oxidation reactions (1 mg brain protein/ml) were performed at 37 °C in buffer B (50 mM sodium phosphate, pH 7.4). When indicated, buffer B was supplemented with 25 mM $NaHCO_3$. Reactions were terminated by addition of 0.2 mM DTPA (pH 7.4), 300 nM catalase, and 0.1 mM BHT. Proteins were precipitated with ice-cold trichloroacetic acid (10% final concentration), acid-hydrolyzed, and subjected to GC/MS analysis. $ONOO^-$ was synthesized from 2-ethoxyethyl nitrite and H_2O_2 (31) and stored at −80 °C. $ONOO^-$ was thawed immediately prior to use, and its concentration was determined spectrophotometrically (ϵ_{302} = 1, 670 M^{-1} cm^{-1}; Ref. 32).

Amino Acid Isolation and Derivatization—All procedures were carried out at 4 °C. Brain tissue (~ 10 mg wet weight) was homogenized in 1 ml of antioxidant buffer. After dialysis overnight against buffer A, samples were delipidated by extraction with water/methanol/water-washed diethyl ether (1:3:7; v/v/v) for 10 min. Protein precipitate was recovered by centrifugation at 500 × *g* for 10 min. The resulting protein pellet was lipid extracted once more and dried under N_2. Samples (~1 mg of protein) were dried under vacuum in 1-ml glass reaction vials and immediately suspended in 0.5 ml of 6 N HCl (Sequenal grade, Pierce) containing 0.1% benzoic acid and 0.1% phenol (w/v). Isotopically labeled internal standards were added, and samples were hydrolyzed at 110 °C for 24 h under argon. Aromatic amino acids were isolated using a solid-phase C-18 column (3 ml; Supelclean SPE, Supelco Inc., Bellefonte, PA) (21) and converted to *n*-propyl esters by addition of 200 μl of HCl/*n*-propanol (prepared by the addition of one volume of 12 N HCl to three volumes of *n*-propanol) and heating for 1 h at 65 °C. After evaporation of excess reagent under N_2, heptafluorobutyric anhydride/ethyl acetate (1:3, v/v) was added, and the samples were heated at 65 °C for 15 min.

Mass Spectrometric Analysis—Amino acids were quantified using isotope dilution negative-ion electron capture GC/MS using a Hewlett Packard 5890 gas chromatograph interfaced with a Hewlett Packard 5988A mass spectrometer with extended mass range (21). Under these chromatography conditions, authentic compounds and isotopically labeled standards exhibited retention times identical to those of analytes derived from tissue samples. The limit of detection (signal/noise >10) was <1 pmol for all of the amino acids. The ions used for detecting analyte and internal standard were: phenylalanine, *m/z* 383 and 389 ions; tyrosine, *m/z* 417 and 423 ions; 3-nitrotyrosine, *m/z* 464 and 470 ions; *ortho*-tyrosine, *m/z* 595 and 601 ions; *o,o'*-dityrosine *m/z* 1208 and 1220 ions.

Statistical Analysis—Differences between two groups were compared using an unpaired Student's *t* test. Multiple comparisons were performed using a two-way analysis of variance. The null hypothesis was rejected at the 0.05 level.

RESULTS

Mass Spectrometric Detection of 3-Nitrotyrosine, o,o'-Dityrosine, and ortho-Tyrosine in Mouse Brain—To determine whether oxidized amino acids are present in normal mouse brain, we assayed freshly isolated tissue from frontal cortex, cerebellum, ventral midbrain and striatum. When amino acids from acid hydrolysates of each tissue were isolated and derivatized with *n*-propanol and heptafluorobutyric anhydride and then analyzed by GC/MS in the negative-ion electron capture mode, we detected compounds that exhibited major ions and retention times identical to those of authentic 3-nitrotyrosine, *ortho*-tyrosine, and *o,o'*-dityrosine. Selected ion monitoring demonstrated that the ions derived from the amino acids co-eluted with ions derived from authentic ^{13}C-labeled internal

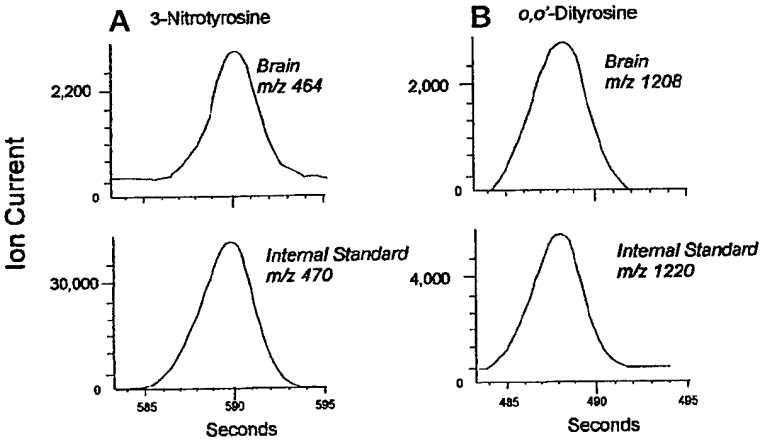

FIG. 1. **Detection of *n*-propyl heptafluorobutyryl derivatives of 3-nitrotyrosine (*A*) and *o,o'*-dityrosine (*B*) in mouse ventral midbrain by selected ion monitoring negative-ion electron capture GC/MS.** Tissue samples were delipidated, acid-hydrolyzed, and subjected to solid-phase extraction on a C-18 column. Isolated oxidized amino acids were derivatized and subjected to GC/MS analysis as described under "Experimental Procedures." Note co-elution of the major ion expected for (*A*) 3-nitrotyrosine (*m/z* 464) with that of 3-nitro[$^{13}C_6$]tyrosine (*m/z* 470) and (*B*) *o,o'*-dityrosine (*m/z* 1208) with that of *o,o'*-[$^{13}C_{12}$]dityrosine (*m/z* 1220).

standards, as shown in Fig. 1 for 3-nitrotyrosine and *o,o'*-dityrosine.

Levels of the oxidized amino acids varied both in absolute magnitude and in different parts of the brain (Fig. 2–4). All regions demonstrated relatively high levels of *ortho*-tyrosine (~ 0.5–1 mmol/mol of phenylalanine), which were highest in the striatum. In contrast, levels of 3-nitrotyrosine and *o,o'*-dityrosine were lower than those of *ortho*-tyrosine, and unlike *ortho*-tyrosine levels, those of *o,o'*-dityrosine were lowest in the striatum. However, 3-nitrotyrosine levels were comparable in all areas of the brain. These results indicate that acid hydrolysates of normal brain tissue proteins contain detectable levels of oxidized amino acids.

3-Nitrotyrosine Is Elevated in Ventral Midbrain and Striatum of MPTP-treated Mice—To determine whether MPTP promotes oxidative damage in brain proteins, we analyzed samples from eight control and eight MPTP-treated animals, comparing levels of oxidation products in the ventral midbrain and striatum, two regions that exhibit marked neuronal injury in MPTP-treated mice. We also analyzed samples from the frontal cortex and cerebellum, two regions of the brain that are little affected by MPTP. Tissue was prepared and analyzed by isotope dilution GC/MS as described above.

Levels of 3-nitrotyrosine in the ventral midbrain and striatum of the MPTP-treated animals were markedly higher (110% and 90%, respectively) than in the controls (Fig. 2). In contrast, there was no difference in level of 3-nitrotyrosine in the frontal cortex or cerebellum. These results indicate that levels of 3-nitrotyrosine increase selectively in the regions of the brain that are susceptible to the neurotoxic action of MPTP.

o,o'-Dityrosine Is Elevated in Ventral Midbrain and Striatum of MPTP-treated Mice—Levels of *o,o'*-dityrosine showed a strikingly similar pattern of increase as 3-nitrotyrosine (elevations of 120% and 170% compared with controls) in ventral midbrain and striatum, two regions of the brain that are vulnerable to MPTP-mediated neurotoxicity (Fig. 3). As with 3-nitrotyrosine, there was no difference in levels of *o,o'*-dityrosine in the frontal cortex or cerebellum. These results indicate that levels of *o,o'*-dityrosine increase selectively in the regions of the brain that are vulnerable to MPTP-mediated neuronal injury.

ortho-Tyrosine Levels Are Unchanged in All Regions of the Brain in MPTP-treated Mice—In contrast to 3-nitrotyrosine and *o,o'*-dityrosine, there was no change in the levels of *ortho*-tyrosine in any of the regions of the brain examined (Fig. 4). Collectively, these results indicate that levels of 3-nitrotyrosine and *o,o'*-dityrosine increase selectively in the regions of the brain that are susceptible to the neurotoxic action of MPTP. In contrast, *ortho*-tyrosine does not accumulate in increased amounts in any region of the brain examined in MPTP-treated animals.

3-Nitrotyrosine, o,o'-Dityrosine, and ortho-Tyrosine in Brain Tissue Oxidized by Peroxynitrite in Vitro—To evaluate the potential usefulness of 3-nitrotyrosine, *ortho*-tyrosine and *o,o'*-dityrosine as markers for oxidation *in vitro*, we investigated the product yield of these compounds in brain proteins that had been oxidized *in vitro* by $ONOO^-$, tyrosyl radical, and $HO^\cdot$. Protein used for the *in vitro* studies was isolated by centrifugation (10,000 × *g* for 10 min) from homogenate prepared from different regions of the brain. In brain proteins exposed to 1 mM $ONOO^-$, there was a dramatic increase (~ 80-fold) in 3-nitrotyrosine (Fig. 5*A*). Proteins isolated from different regions of the brain demonstrated similar increases after they were oxidized *in vitro* with $ONOO^-$. When brain protein was added 2 min after $ONOO^-$, however, protein nitration was minimal, indicating that $ONOO^-$ or a short-lived species derived from $ONOO^-$ nitrates the aromatic ring of protein-bound tyrosine (data not shown).

Because $ONOO^-$ also hydroxylates aromatic compounds and promotes cross-linking of phenolic groups (33), we determined whether *ortho*-tyrosine and *o,o'*-dityrosine form in brain proteins exposed to $ONOO^-$. Levels of *ortho*-tyrosine and *o,o'*-dityrosine increased 2–3-fold when we exposed brain protein to this reactive nitrogen species, but the product yields of *o,o'*-dityrosine and *ortho*-tyrosine were <5% and <25% that of 3-nitrotyrosine (Fig. 5, *B* and *C*). *In vitro* studies of model proteins exposed to a wide variety of different oxidation systems have previously demonstrated that 3-nitrotyrosine is a specific marker for protein oxidation by reactive nitrogen species (34).

Recent studies indicate that $ONOO^-$ rapidly reacts with carbon dioxide to generate $ONO_2CO_2^-$ (35–41). Bicarbonate is in equilibrium with carbonic acid that is present in extracellular fluids primarily as carbon dioxide, its conjugate acid. Bicarbonate/carbon dioxide is present at high concentrations *in vivo*, and the reactivity of $ONO_2CO_2^-$ differs from that of $ONOO^-$ (35–37). We therefore determined whether the addition of 25 mM $NaHCO_3$ to the reaction buffer affected the product yields of oxidized amino acids in brain proteins exposed to $ONOO^-$. The absolute increases and relative yields of 3-nitrotyrosine, *o,o'*-dityrosine, and *ortho*-tyrosine in brain proteins exposed to $ONOO^-$ were almost identical in the presence and absence of added bicarbonate/carbon dioxide (Fig. 5). The failure of $NaHCO_3$ to affect the product yields of the oxidized amino acids likely reflects the presence of bicarbonate in brain proteins and/or reaction mixture used for the experiments (35–41).

Taken together, these results indicate that 3-nitrotyrosine is

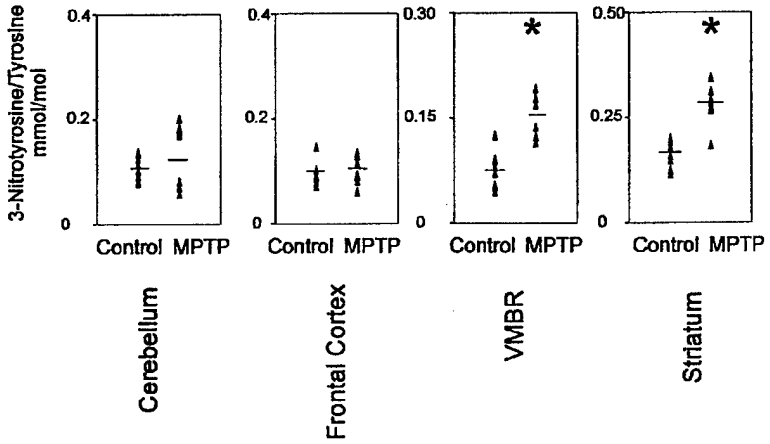

FIG. 2. **3-Nitrotyrosine in brain proteins isolated from control and MPTP-treated mice.** After addition of ^{13}C-labeled internal standards, tissue from the indicated region of the brain was delipidated, hydrolyzed, and subjected to solid-phase extraction. The isolated amino acids were derivatized and analyzed by negative-ion electron capture GC/MS with selected ion monitoring. Three independent analyses of tissue were performed on 8 control and 8 MPTP-treated animals for a total of 24 analyses for each area of the brain. Values are the mean ± S.E. and are normalized to tyrosine and phenylalanine, the precursor amino acids. *, $p < 0.05$ by analysis of variance.

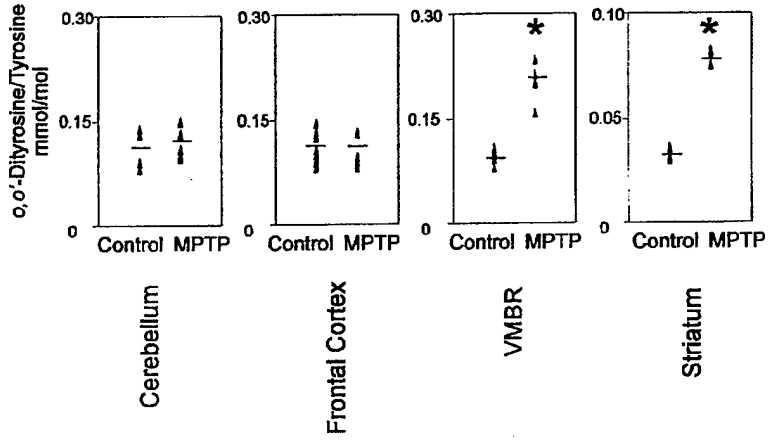

FIG. 3. **o,o'-Dityrosine in brain proteins isolated from control and MPTP-treated mice.** Tissue from the indicated region of brain in control and MPTP-treated mice was subjected to isotope dilution GC/MS analysis. Values are the mean ± S.E. of triplicate determinations from 8 control animals and 8 MPTP-treated animals. *, $p < 0.05$ by analysis of variance.

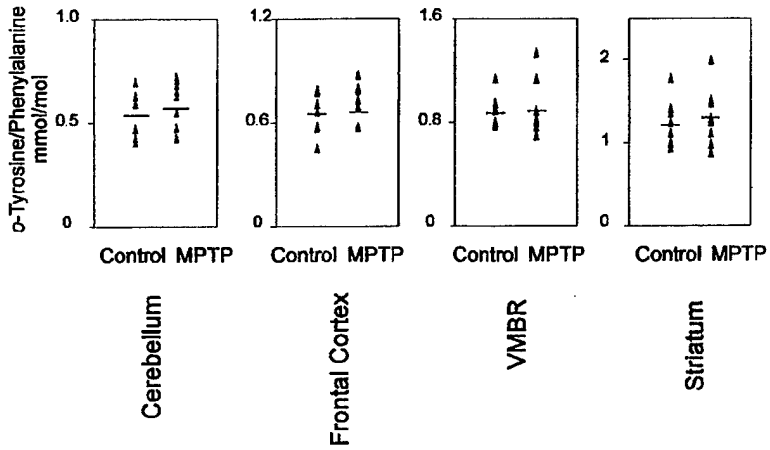

FIG. 4. ***ortho*-Tyrosine in brain proteins isolated from control and MPTP-treated mice.** Tissue from the indicated region of brain in control and MPTP-treated mice was subjected to isotope dilution GC/MS analysis. Values are the mean ± S.E. of triplicate determinations from 8 control animals and 8 MPTP-treated animals.

an excellent marker for proteins oxidized by reactive nitrogen species *in vitro*. They also suggest that *ortho*-tyrosine is a significant product of protein oxidation by $ONOO^-$. In contrast, *o,o'*-dityrosine is a relatively minor product.

3-Nitrotyrosine, o,o'-Dityrosine, and ortho-Tyrosine in Brain Protein Oxidized in Vitro by Myeloperoxidase-generated Tyrosyl Radical—We exposed protein derived from various areas of the brain to tyrosyl radical generated by the myeloperoxidase-tyrosine-H_2O_2 system, using physiologically plausible levels of oxidant and substrate (0.1 mM H_2O_2 and 0.2 mM tyrosine; Ref. 21). *o,o'*-Dityrosine was the major product of the reaction, with no change in levels of either 3-nitrotyrosine or *ortho*-tyrosine (Fig. 6). All regions of the brain exposed to tyrosyl radical showed a similar increase in *o,o'*-dityrosine. These results indicate that tyrosyl radical generated by myeloperoxidase selectively raises *o,o'*-dityrosine levels without changing levels of 3-nitrotyrosine or *ortho*-tyrosine.

Recent studies indicate that the myeloperoxidase-H_2O_2 system will convert tyrosine into 3-nitrotyrosine in a reaction that requires nitrite, (33) a degradation product of NO. Brain proteins incubated with myeloperoxidase, 0.1 mM H_2O_2, and 50 μM nitrite had 3-nitrotyrosine levels similar to those observed in MPTP-treated mice (Table I). These observations indicate that myeloperoxidase can promote the formation of *o,o'*-dityrosine and 3-nitrotyrosine by two distinct pathways, one involving tyrosyl radical and the other involving reactive nitrogen species.

3-Nitrotyrosine, o,o'-Dityrosine, and ortho-Tyrosine in Brain

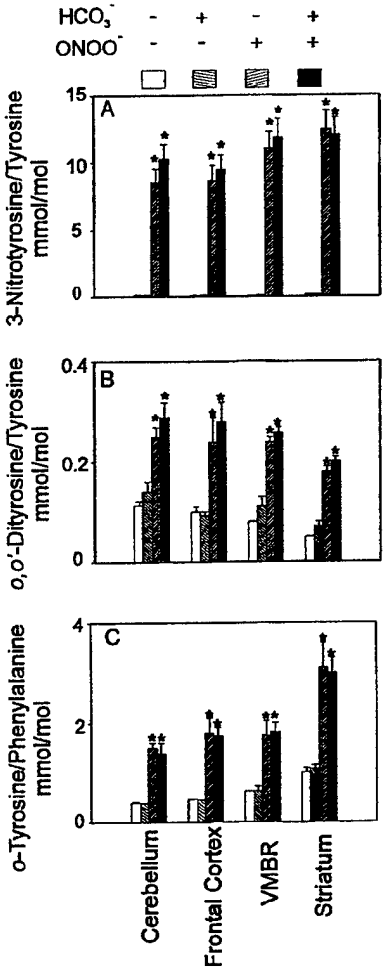

FIG. 5. **Product yields of 3-nitrotyrosine (*A*), *o,o'*-dityrosine (*B*), and *ortho*-tyrosine (*C*) in brain proteins oxidized *in vitro* by peroxynitrite.** Proteins from the indicated areas of the brain were incubated for 10 min at 37 °C in buffer B alone (50 mM sodium phosphate, pH 7.4), buffer B with 25 mM HCO_3^-, buffer B supplemented with 1 mM $ONOO^-$, or buffer B with 1 mM $ONOO^-$ and 25 mM HCO_3^-. Reactions were initiated by the addition of $ONOO^-$. After acid precipitation and addition of ^{13}C-labeled internal standards, proteins were hydrolyzed and subjected to solid-phase extraction. The isolated amino acids were derivatized and analyzed by isotope dilution negative-ion electron capture GC/MS with selected ion monitoring. Values are the mean ± S.E. of triplicate determinations and are normalized to levels of precursor amino acid. Similar results were observed in two independent experiments. *, $p < 0.01$ by paired *t* test.

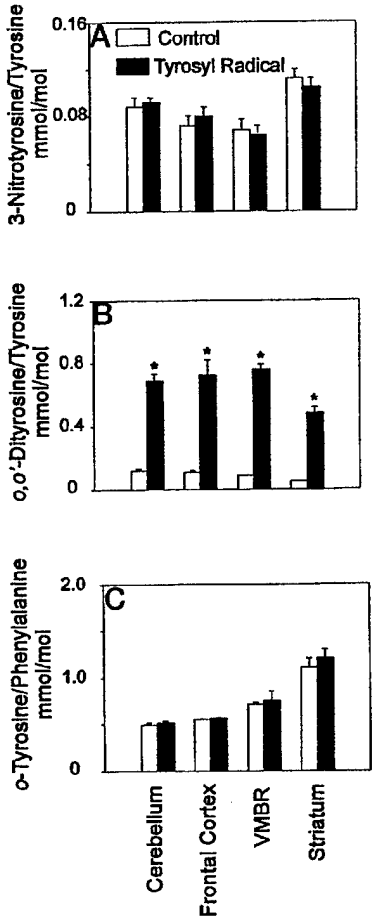

FIG. 6. **Product yields of 3-nitrotyrosine (*A*), *o,o'*-dityrosine (*B*), and *ortho*-tyrosine (*C*) in brain proteins oxidized *in vitro* by myeloperoxidase-generated tyrosyl radical.** Proteins from the indicated areas of the brain were incubated for 30 min at 37 °C in buffer B containing 0.1 mM DTPA (*Control*) or buffer B supplemented with of 0.1 mM H_2O_2, 20 nM myeloperoxidase, 0.2 mM L-tyrosine, and 0.1 mM DTPA (*Tyrosyl Radical*). Levels of oxidized amino acids in tissue proteins were determined by isotope dilution GC/MS. Values are the mean ± S.E. of triplicate determinations and are normalized to levels of precursor amino acid. Similar results were observed in two independent experiments. *, $p < 0.01$.

Protein Oxidized by Hydroxyl Radical in Vitro—Proteins isolated from the cerebellum, frontal cortex, ventral midbrain, and striatum of control mice were incubated with a $HO^\cdot$-generating system (copper plus H_2O_2). We then determined levels of 3-nitrotyrosine, *o,o'*-dityrosine, and *ortho*-tyrosine in amino acid hydrolysates of the proteins using isotope dilution GC/MS. *ortho*-Tyrosine and *o,o'*-dityrosine accumulated in all four areas of the brain (Fig. 7, *A* and *B*). In contrast, the level of 3-nitrotyrosine did not change (Fig. 7*C*). The absolute increase in the level of *ortho*-tyrosine was ~10-fold higher than that of *o,o'*-dityrosine. It should be noted that copper plus H_2O_2 is a metal-catalyzed oxidation system that generates other reactive species in addition to $HO^\cdot$ (42, 43). *In vitro* studies have demonstrated, however, that the relative product yields of *o,o'*-dityrosine and *ortho*-tyrosine are similar in model proteins exposed to copper plus H_2O_2 or ionizing radiation (a relatively pure source of $HO^\cdot$; Ref. 44). These observations suggest that *ortho*-tyrosine might serve as a marker for protein damage by $HO^\cdot$ *in vivo*.

TABLE I

Product yield of 3-nitrotyrosine in brain proteins oxidized in vitro by the myeloperoxidase-H_2O_2-nitrite system

Proteins from the indicated area were incubated for 30 min at 37 °C in buffer B supplemented with 0.1 mM DTPA (Control) or buffer B supplemented with 20 nM myeloperoxidase (MPO), 0.1 mM H_2O_2, 50 μM nitrite, and 0.1 mM DTPA. Levels of protein-bound 3-nitrotyrosine were determined using isotope dilution GC/MS. Values are normalized to the content of the precursor amino acid tyrosine. Values are the mean ± S.E. of three determinations. Similar results were observed in two independent experiments. *, $p < 0.01$.

Brain area	3-Nitrotyrosine	
	Control	MPO-H_2O_2-nitrite
	μmol/mol	
Cerebellum	94 ± 16	313 ± 67*
Frontal cortex	79 ± 13	182 ± 16*
Ventral midbrain	84 ± 12	268 ± 60*
Striatum	195 ± 28	413 ± 42*

The concentrations of oxidant and redox catalysts were very different in the $HO^\cdot$ and tyrosyl radical systems. There was 50

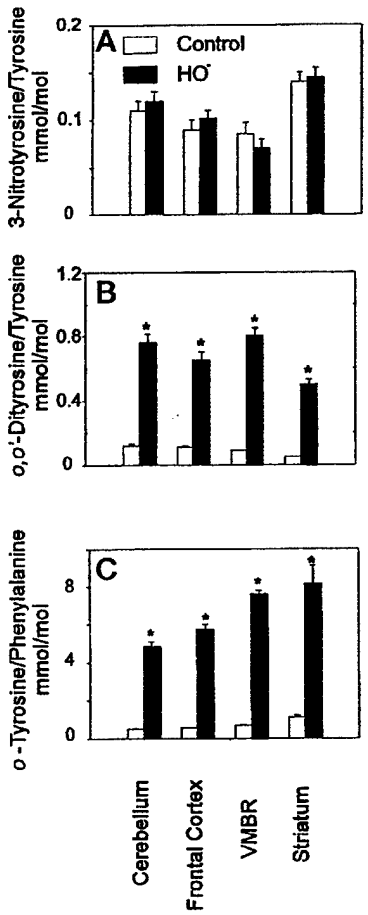

FIG. 7. **Product yields of 3-nitrotyrosine (*A*), *o,o'*-dityrosine (*B*), and *ortho*-tyrosine (*C*) in brain proteins oxidized *in vitro* by hydroxyl radical.** Proteins from the indicated areas of the brain were incubated for 2 h at 37 °C in buffer B (*Control*) or buffer B supplemented with 0.2 mM $CuSO_4$ and 5 mM H_2O_2 ($HO^{\cdot}$). To inhibit residual tissue catalase activity, 0.1 mM 3-amino-1,2,4-triazole was included in reaction mixtures. Levels of oxidized amino acids in tissue proteins were determined by isotope dilution GC/MS. Values are the mean ± S.E. of triplicate determinations and are normalized to levels of precursor amino acid. Similar results were observed in two independent experiments. *, $p < 0.01$.

times as much H_2O_2 in the $HO^{\cdot}$ system as in the tyrosyl radical system (5 mM *versus* 0.1 mM), and 10,000 times as much copper as myeloperoxidase (200 μM *versus* 20 nM). In addition, proteins were exposed to the $HO^{\cdot}$ system for 4 times longer than the tyrosyl radical system. Despite these marked differences, the relative yields of *o,o'*-dityrosine were similar when brain protein was exposed to either $HO^{\cdot}$ or tyrosyl radical. These observations imply that, under our experimental conditions, myeloperoxidase-generated tyrosyl radical is much more efficient than $HO^{\cdot}$ at producing *o,o'*-dityrosine.

DISCUSSION

In the present studies, we used highly sensitive and specific isotope dilution mass spectrometric methods to investigate the pathways that oxidatively damage proteins in the brain under normal and pathological situations. We focused on three oxidants of potential pathophysiological significance: $ONOO^-$, a product of the interaction between O_2^- and NO; tyrosyl radical generated by the heme protein myeloperoxidase; and $HO^{\cdot}$ radical generated by a metal-catalyzed oxidation system. We chose 3-nitrotyrosine, *ortho*-tyrosine, and *o,o'*-dityrosine as markers of protein oxidation because they are stable to acid hydrolysis and likely represent post-translational modifications of proteins.

In normal mice, we found detectable levels of 3-nitrotyrosine, *ortho*-tyrosine, and *o,o'*-dityrosine in all brain regions studied. This finding is consistent with the view that basal levels of oxidatively damaged proteins exist in physiological situations, especially in the brain. They probably result from the combination of the high rate of oxygen consumption and the poor oxidant-scavenging arsenal that characterize this organ. This "basal oxidative stress" seems to affect not only proteins, but DNA as well, since significant levels of 8-hydroxy-deoxyguanosine, a marker of oxidative damage to DNA, are present in normal rodent and human brains (45, 46). Collectively, these data support the idea that a mild but persistent oxidative stress may be normal and may lead, over time, to the decline in physiological functions that characterize aging (47). Interestingly, levels of the oxidation markers we assayed were not constant throughout the brain. In fact, we observed significant variations in 3-nitrotyrosine and *ortho*-tyrosine levels among the different brain regions studied, with highest levels in the striatum. The striatum also was among the brain regions showing the highest levels of 8-hydroxy-deoxyguanosine in both rats and humans (45, 46). These findings are in keeping with the belief that the striatum, and more broadly, the basal ganglia, are vulnerable to oxidative stress and frequently affected in oxidant-related neurodegenerative disorders (3).

Compared with saline-treated controls, the brains of MPTP-treated mice exhibited strikingly elevated levels of both *o,o'*-dityrosine and 3-nitrotyrosine. Moreover, we observed significant increases in these two markers in striatum and in ventral midbrain but not in frontal cortex and cerebellum, which is consistent with the specificity of MPTP's neurotoxic effects on the nigrostriatal dopaminergic pathway. These observations indicate that MPTP promotes protein oxidation specifically in brain regions known to be affected by the toxin and provide evidence that it stimulates both the production of reactive species and oxidative damage to critical cellular elements.

ortho-Tyrosine was the major product when brain proteins were exposed *in vitro* to $HO^{\cdot}$ generated by a metal-catalyzed oxidation system. In contrast, *ortho*-tyrosine concentrations were not altered in brain tissue from MPTP-treated mice. MPTP has been proposed to enhance $HO^{\cdot}$ production by impairing mitochondrial respiration (17, 48, 49). However, the lack of a detectable increase in *ortho*-tyrosine levels in the brain of MPTP-treated mice argues against a prominent role for $HO^{\cdot}$ in protein damage and casts doubt on the participation of $HO^{\cdot}$ in MPTP-induced neurotoxicity *in vivo*.

The marked increase in 3-nitrotyrosine levels after MPTP administration strongly suggests that reactive nitrogen intermediates play key roles in protein oxidation. Compelling evidence for this view comes from our *in vitro* experiments, which show that $ONOO^-$ causes a striking elevation of 3-nitrotyrosine, with a significant increase in *ortho*-tyrosine and a much smaller increase in *o,o'*-dityrosine. Our observations are in good agreement with previous demonstrations that inhibition of nitric-oxide synthase attenuates MPTP-induced dopaminergic toxicity (16–18).

Beal and collaborators showed that MPTP significantly increases striatal levels of free 3-nitrotyrosine in mice (17). However, the relationship between free 3-nitrotyrosine and 3-nitrotyrosine in proteins is unknown, and the pathophysiological significance, if any, of free 3-nitrotyrosine remains to be determined. In contrast, protein nitration may be deleterious because it alters the pK_a of tyrosine and can affect the protein's secondary and tertiary organization, thereby altering its function, as we have demonstrated for tyrosine hydroxylase (30).

Therefore, our data not only confirm that MPTP increases brain levels of 3-nitrotyrosine, but, more importantly, they strongly support the hypothesis that tyrosine nitration of brain proteins may play a critical role in the MPTP neurotoxic process.

The *o,o'*-dityrosine increase we observed after MPTP administration suggests that a pathway involving tyrosyl radical may provide a second physiologically relevant mechanism for protein oxidation in this model. In all cases where the biochemical pathway is known, a heme protein mediates *o,o'*-dityrosine synthesis. One such candidate is myeloperoxidase, which is specifically expressed by myeloid cells and may be present at low levels in brain macrophages and microglial cells. It also appears to be up-regulated in activated macrophage/microglial cells around demyelinating lesions in multiple sclerosis (50).

Myeloperoxidase uses H_2O_2 to generate oxidizing intermediates that destroy invading pathogens. Recent studies indicate that this enzyme and other heme proteins can use H_2O_2 to oxidize nitrite, a decomposition product of NO, generating 3-nitrotyrosine and other nitration products *in vitro* (33, 51–53). Heme proteins also oxidize tyrosine to tyrosyl radical, a reactive intermediate that promotes *o,o'*-dityrosine formation. Brain proteins incubated with the myeloperoxidase-nitrite-H_2O_2 system demonstrated an increase in 3-nitrotyrosine levels similar to those observed in MPTP-treated mice. These observations indicate that myeloperoxidase can promote the formation of *o,o'*-dityrosine and 3-nitrotyrosine by two distinct pathways, one involving tyrosyl radical and the other involving reactive nitrogen species. In light of these *in vitro* oxidation patterns and the protective effects of nitric-oxide synthase inhibitors, we speculate that if myeloperoxidase or other heme proteins contribute to the neurotoxicity of MPTP, they act through the pathway involving reactive nitrogen species.

In the MPTP model, a strong astrocytic and microglial reaction occurs in both the striatum and ventral midbrain soon after dopamine neurons begin to die (54). This robust glial reaction might provide the necessary cellular substrate for myeloperoxidase induction and therefore for *o,o'*-dityrosine production. According to this scenario, damage inflicted by tyrosyl radical may be a secondary event in MPTP-induced neurotoxicity. This does not, however, undermine the pathological significance of myeloperoxidase action, which would peak during the most active phase of neurodegeneration (55). Therefore, tyrosyl radical may not initiate but may enhance dopaminergic neuronal death in the MPTP mouse model of PD. Other heme proteins that can catalyze the selective increase in 3-nitrotyrosine and *o,o'*-dityrosine in the brains of MPTP-treated mice include mitochondrial heme proteins like cytochrome *c*. The latter might be especially relevant to both MPTP and PD because a large body of evidence points to the mitochondrion as the site of the major deleterious event that drives dopaminergic neurodegeneration.

The overall increase in oxidized amino acids in MPTP-induced brain injury represents ~2 in 10,000 protein tyrosine residues. One could raise the question of whether this low level of oxidation products is likely to be biologically important. We believe that the increases in specific oxidation products we observe may be significant for several reasons. First, similar levels of oxidation products have been reported in other conditions where oxidative damage has been implicated in the pathogenesis of disease (21, 34, 56–62). Second, an inherent problem with the analysis of biological material is that there is substantial "dilution" of the targets of protein oxidation with proteins present in surrounding normal tissue. Therefore, even with substantial damage to specific proteins it is conceivable that the overall level of protein oxidation products will be low. Indeed, the selective nitration of tyrosine hydroxylase by reactive nitrogen species has been proposed to be of critical pathologic importance in the pathogenesis of PD (30). Third, there could be other targets for damage by reactive oxidant species such as lipids and DNA that are biologically relevant. In this sense, the products we have quantified may be serving as markers of oxidative damage. Finally, we believe that our *in vitro* studies provide strong evidence that the pattern of elevation of oxidized amino acids serves as a "molecular" fingerprint for the pathways that are mediating oxidative damage *in vivo* (19, 63). Though the oxidation products themselves may or may not be pathophysiologically significant, the increases in 3-nitrotyrosine and *o,o'*-dityrosine we observe point toward reactive nitrogen species and tyrosyl radical as being physiologically relevant. In contrast, there is no evidence that HO$^\cdot$ (the oxidant generally proposed to be mediating protein damage) is playing a role in this model of PD.

Because of the similarity between the MPTP model and PD, it is possible that the culprits identified above may underlie the oxidative attack that presumably kills dopaminergic neurons in PD. Accordingly, the present study may have important implications for understanding the pathogenesis of PD and for developing antioxidant interventions to halt or retard neurodegeneration in this disorder.

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Poly(ADP-ribose) polymerase activation mediates 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism

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ABSTRACT **1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin that causes parkinsonism in humans and nonhuman animals, and its use has led to greater understanding of the pathogenesis of Parkinson's disease. However, its molecular targets have not been defined. We show that mice lacking the gene for poly(ADP-ribose) polymerase (PARP), which catalyzes the attachment of ADP ribose units from NAD to nuclear proteins after DNA damage, are dramatically spared from MPTP neurotoxicity. MPTP potently activates PARP exclusively in vulnerable dopamine containing neurons of the substantia nigra. MPTP elicits a novel pattern of poly(ADP-ribosyl)ation of nuclear proteins that completely depends on neuronally derived nitric oxide. Thus, NO, DNA damage, and PARP activation play a critical role in MPTP-induced parkinsonism and suggest that inhibitors of PARP may have protective benefit in the treatment of Parkinson's disease.**

Parkinson's disease (PD) is a common and disabling idiopathic neurodegenerative disorder characterized by tremor, bradykinesia, rigidity, and balance difficulties. These motor abnormalities are attributed to depletion of brain dopamine (DA) that results from the dramatic loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (1–3). Although there are therapies available for PD that help alleviate symptoms, they may produce major side effects and lose efficacy over time as they do not modify the progressive neurodegeneration in PD (4). Insight into the neurodegenerative process in PD comes from the use of the selective neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which replicates parkinsonian motor signs in human and nonhuman animals (5–11). As in PD, MPTP can produce loss of dopaminergic neurons within the SNpc, Lewy body-like intraneuronal eosinophilic inclusions, markers of increased oxidative stress, and decrements in mitochondrial complex I activity (5–8). Recent studies suggest that nitric oxide (NO) and superoxide anion (O_2^-) may play a role in MPTP neurotoxicity through mechanisms that are not known (12–16). Peroxynitrite is thought to play a prominent role in O_2^- and NO-mediated neurotoxicity, which can result in cell death with both apoptotic and nonapoptotic morphologies (17–19). NO, O_2^-, and peroxynitrite have vast potential targets, but share at least one common downstream target in that they damage DNA (17–20). DNA damage is a prime activator of poly(ADP-ribose) polymerase (PARP, EC 2.4.4.30), which uses NAD as a substrate to transfer ADP ribose groups to a variety of nuclear proteins. PARP is activated by binding to DNA ends or strand breaks, and its activity is strictly proportional to the number of DNA breaks, whereas it is totally inactive in the absence of DNA breaks (20–25). One of the earliest nuclear events that follows DNA strand breakage in response to exposure to free radicals is the poly(ADP-ribosyl)ation of nuclear proteins that are localized predominantly adjacent to the DNA strand breaks. Although PARP is a prominent caspase cleavage target during apoptosis, it is unlikely to play a prominent role in apoptotic cell death as cells lacking the gene for PARP are equally susceptible to apoptosis induced by tumor necrosis factor α, Fas ligand, or γ-irradiation (26, 27). Furthermore, PARP is activated primarily by single-strand nicks of DNA that typically occur after free radical damage, but it is insensitive to double-strand DNA ends that typically occur during apoptosis (28). The exact function of PARP is not clear, but it is thought to play accessory roles in DNA replication, genomic stability, recombination, and DNA repair (20–23, 29, 30). Although massive activation of PARP in acute injury of neurons during stroke leads to cell death, most likely through energy depletion (31–34), its potential role in a chronic neurodegenerative process is not known. In the present study we show that PARP activation is instrumental in MPTP-induced parkinsonism and dopaminergic neuronal loss.

METHODS

Animals. All experiments were approved and conformed to the guidelines set by the Institutional Animal Care Committee. To avoid differences caused from strain effect or divergent genetic lines, *PARP*$^{-/-}$ mice used in this study were on a pure 129 Sv/Ev background (26) with the colony maintained by outbreeding with purebred 129 Sv/Ev wild-type (WT) controls (Taconic Farms). Thus the *PARP*$^{-/-}$ mice are of the same strain as controls, and inbreeding effects are minimized. All mice were also age- and gender-matched (male, 60–90 days old) to avoid known effects of age and estrogen on MPTP-induced neurotoxicity.

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This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PARP, poly(ADP-ribose) polymerase; PD, Parkinson's disease; DA, dopamine; SNpc, substantia nigra pars compacta; DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; TH, tyrosine hydroxylase; PB, phosphate buffer; WT, wild type; MPP^+, 1-methyl-4-phenylpyridimium; MAO-B, monoamine oxidase B; nNOS, neuronal NO synthase.

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MPTP Treatment. *PARP*$^{-/-}$ and WT mice were housed three to a cage in a temperature-controlled room with 12-hr dark/light cycle and free access to food and water for the duration of the experiment. Each mouse received four i.p. injections of MPTP-HCl (20 mg/kg free base, Research Biochemicals) in saline or saline alone at 2-hr intervals.

Measurement of Striatal DA, Dihydroxyphenylacetic Acid (DOPAC), and Homovanillic Acid (HVA) Levels. HPLC with electrochemical detection was used to measure striatal levels of DA, DOPAC, and HVA (14, 15). Seven days after the last MPTP injection, mice (4–6 per group) were sacrificed, brains were quickly removed, and striata were dissected out on an ice-cold glass Petri dish (35). Samples were immediately frozen on dry ice and stored at −80°C until analysis. On the day of the assay, tissue samples were sonicated in 50 volumes of 0.1 M perchloric acid containing 25 μg/ml of dihydrobenzylamine (Sigma) as internal standard. After centrifugation (15,000 × *g*, 10 min, 4°C), 20 μl of supernatant was injected onto a C18-reversed phase RP-80 catecholamine column (ESA, Bedford, MA). The mobile phase consisted of 90% of a solution of 50 mM sodium phosphate, 0.2 mM EDTA, and 1.2 mM heptanesulfonic acid (pH = 3.5) and 10% methanol. Flow rate was 1.0 ml/min. Peaks were detected by a Coulochem 5100A detector (E1 = −0.04 V, E2 = + 0.35 V) (ESA). Data were collected and processed on a computerized Dynamax data manager (Rainin Instruments).

Measurement of Striatal l-Methyl-4-Phenylpyridimium (MPP^+) Levels. HPLC with UV detection (wavelength 295 nm) was used to measure striatal MPP^+ levels (14). *PARP*$^{-/-}$ and WT mice (three per time point) were sacrificed 90, 120, and 240 min after the fourth MPTP injection. Striata were dissected as above, immediately frozen, and stored at −80°C until analysis. On the day of the assay, tissue samples were sonicated in 5 vol of 5% tricholoracetic acid containing 5 μg/ml of 4-phenylpyridine (Sigma) as internal standard. After centrifugation (as for catecholamines), 50–100 μl of supernatant was injected onto a cation-exchange Ultracyl-CS column (Beckman, San Ramon, CA). The mobile phase consisted of 90% of a solution of 0.1 M acetic acid and 75 mM triethylamine-HCl (pH 2.35 adjusted with formic acid), and 10% acetonitrile. The flow rate was 1.5 ml. Data were collected and processed as above.

Immunohistochemistry. For tyrosine hydroxylase (TH) immunohistochemistry and Nissl staining, at 7 days after the last dose of MPTP, mice (five per group) were perfusion-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4 as described (15). After postfixation in the same fixative solution, and cryoprotection in 20% sucrose/PB, brains were frozen and serially sectioned (30 μm for TH) through the entire midbrain. Alternate sections were stained for Nissl or TH. Neurons containing TH were demonstrated by incubating the tissue sections successively with a rabbit polyclonal anti-TH antibody (1:1,000, Eugene Tech, Ridgefield Park, NJ), a biotinylated–conjugated polyclonal goat anti-rabbit antibody (1:200; Vector Laboratories), and a horseradish-peroxidase-conjugated avidin/biotin complex (Vector) as described (15, 36).

Poly(ADP-Ribose) Polymer Western Blots and Immunohistochemistry. Ventrolateral midbrain and striata were dissected from mice treated with MPTP and immediately frozen. Samples were homogenized in buffer (sucrose/DTT) and centrifuged (5 min, 14,000 × *g*), and the pellet was resuspended in buffer. Protein concentrations were determined by the Bradford assay, and equal samples were loaded on a gradient SDS/PAGE (30 μg per lane). The gels were transferred to a nitrocellulose membrane and incubated with anti-poly(ADP-ribose) mAb. Membranes were stained with Ponceau S (0.1%) to confirm equal loading and transfer. After blocking of nonspecific sites, membranes were incubated with antibodies to poly(ADP-ribose) (1:250). Bands were visualized via chemiluminesence. For immunohistochemistry mice were perfusion-fixed in 4% paraformaldehyde in 0.1 M PB (pH 7.4) first by deeply anesthetizing the animal with i.p. pentobarbital. Chilled 1× PBS was infused into the left ventricle as blood was allowed to escape the right atrium. Once blood was replaced by PBS, the PB-paraformaldehyde was infused. After infusion, the brain was removed and allowed to postfix in PB-paraformaldehyde for 4 hr and then transferred to 20% glycerol/PB for cryoprotection. The brains were blocked and frozen for sliding microtome sectioning. Serial sections (40 μm) through the midbrain and striatum were taken and incubated with a polyclonal guinea pig antibody to poly(ADP-ribose) (1:400) in PBS-12% BSA overnight at 4°C. Sections were washed for 10 min × 3 in PBS-12% BSA and incubated with secondary anti-guinea pig antibodies (biotin-conjugated, Jackson ImmunoResearch) in PBS-12% BSA at room temperature for 45 min. Texas-red (Vector Laboratories) was used to visualize the immunostaining. Sections were washed for 10 min × 2 in PBS-12% and then for 10 min × 2 in PBS. Slides were mounted with PBS in 80% glycerol and visualized by fluorescence microscopy through a green filter. A black and white camera digitally captured the image to a computer, which then was pseudocolored red (IP Scanalytics, Fairfax, VA).

Stereology. The total number of TH- and Nissl-stained SNpc neurons were counted from five mice per group by using the optical fractionator (37), an unbiased method of cell counting that is not affected by either the volume of reference (i.e., SNpc) or the size of the counted elements (i.e., neurons). Neuronal counts were performed by using a computer-assisted image analysis system consisting of a Zeiss Axiophot photomicroscope equipped with a MC-XYZ-LC (Applied Scientific Instrumentation, Eugene, OR) computer-controlled motorized stage, a DAGE-MTA (Michigan, IN) video camera, a Macintosh 9600 workstation, and NeuroZoom morphometry software (Scripps Research Institute, La Jolla, CA) (38). In agreement with this method, TH- and Nissl-stained neurons were counted in the right and left SNpc of every fourth section throughout the entire extent of the SNpc. Each midbrain section was viewed at low power (×10 objective), and the SNpc was outlined by using the set of anatomical landmarks defined previously (15, 36). Then at a random start, the number of TH- and Nissl-stained cells were counted at high power (×100 oil; numerical aperture 1.4). To avoid double counting of neurons with unusual shapes, TH- and Nissl-stained cells were counted only when their nuclei were optimally visualized, which occurred only in one focal plane. In addition, neurons were differentiated from nonneuronal cells, including glia, in the Nissl stain by the exclusion of cells that did not have a clearly defined nucleus, cytoplasm, and a prominent nucleolus. Although some small neurons may be excluded, these criteria should reliably exclude all non-neuronal cells. After all of the TH- and Nissl-stained neurons were counted, the total numbers of TH- and Nissl-stained neurons in the SNpc were calculated by using the formula described by West *et al.* (37).

Statistical Analysis. Throughout the experiments, the investigators were blinded to the genotype of the mice (i.e., *PARP*$^{-/-}$ or WT) and the treatment received (i.e., MPTP or saline). All values are expressed as the mean ± SEM. Differences among means were analyzed by using one-way or two-way ANOVA. When two-way ANOVA was appropriate, the different genotypes and treatments were used as the independent factors. When ANOVA showed significant differences, pair-wise comparisons between means were tested by Fisher or Newman-Keuls post hoc tests. In all analyses, the null hypothesis was rejected at the 0.05 level. All statistical analyses were performed by using STATVIEW (Abacus Software, San Francisco).

RESULTS

Most pharmacological PARP inhibitors lack specificity and have poor central nervous system bioavailability, raising serious questions about the validity of *in vivo* studies (20–22). To overcome these problems and to ascertain whether PARP activation participates in MPTP neurotoxicity, we examined the effects of MPTP in mice with targeted disruption of the gene that encodes for PARP ($PARP^{-/-}$) (26, 39) compared with strain-, age-, and gender-matched WT controls (Fig. 1). It is important to mention that the $PARP^{-/-}$ mice used in this study are congenic and outbred with the 129 Sv/Ev strain (26), thus any observed phenotype is most likely caused by the absence of the PARP gene and is not the result of genetic strain effects. One week after four injections of MPTP at 20 mg/kg, an 80–90% reduction in striatal DA, DOPAC, and HVA is observed in WT mice (Fig. 1). $PARP^{-/-}$ mice are resistant to the toxic effects of MPTP and show significantly lower reductions in DA, DOPAC, and HVA levels than WT mice (Fig. 1).

In addition to the destruction of DA nerve terminals in the striatum, there is an accompanying loss of DA cell bodies in the SNpc in PD. Furthermore, MPTP-induced destruction of DA nerve terminals does not necessarily equate with loss of cell bodies (40, 41). Thus, it is important to determine whether the absence of PARP protects against the actual loss of DA neurons in the MPTP mouse model. By using stereological techniques, we counted the number of nigral TH-positive neurons in saline-injected WT and $PARP^{-/-}$ mice 1 week after four injections of MPTP at 20 mg/kg (Fig. 2). In WT and $PARP^{-/-}$ mice there is a large number of TH-positive cell bodies intermingled with a dense network of TH-positive nerve fibers within the SNpc, and there is no significant difference in the number of TH-positive cells between the two groups of saline-injected animals (data not shown). MPTP causes a 60% reduction in nigral TH-positive neurons in WT mice compared with saline-injected controls. The loss of SNpc TH-positive neurons after MPTP is virtually abolished in $PARP^{-/-}$ mice (Fig. 2). MPTP also causes a 60% reduction in Nissl-stained SNpc neurons after MPTP in WT mice, whereas Nissl-stained SNpc neurons are completely spared in $PARP^{-/-}$ mice (Fig. 2). The preservation of both TH and Nissl-stained neurons in the $PARP^{-/-}$ mice indicates that the absence of PARP prevents the MPTP-induced death of SNpc neurons.

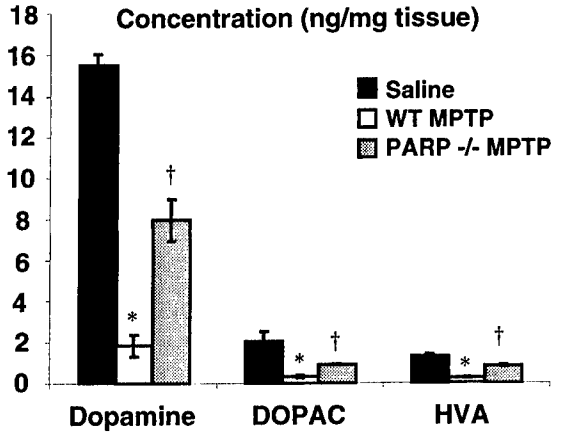

FIG. 1. $PARP^{-/-}$ mice are resistant to the toxic effects of MPTP. HPLC with electrochemical detection of DA and metabolites, HVA and DOPAC, 1 week after MPTP administration. All animals are on a pure genetic background of 129 SvEv. No differences in striatal DA or metabolite content was observed in WT or $PARP^{-/-}$ animals after saline injection, and these values are graphically combined ($n = 10$). WT animals ($n = 8$) injected with MPTP (20 mg/kg ×4) have significant reductions in DA and metabolites compared with saline controls and $PARP^{-/-}$ ($n = 5$). Two-way ANOVA, $P < 0.004$ for DA, HVA, and DOPAC. Student-Newman-Keuls post hoc analysis, *, $P < 0.05$ WT MPTP vs. saline, †, $P < 0.05$ WT MPTP vs. $PARP^{-/-}$ MPTP.

MPTP requires conversion to MPP^+ by monoamine oxidase B (MAO-B) to elicit neurotoxicity (42). MPP^+ then is transported into DA neurons where it concentrates in the mitochondria and inhibits complex I (43–46). To ensure that the protection afforded by the disruption of the PARP gene is not caused by alterations in MPP^+ concentrations in the brain we monitored MAO-B activity and striatal MPP^+ levels in WT and $PARP^{-/-}$ mice. Brain MAO-B activity is not significantly different between WT and $PARP^{-/-}$ mice (Table 1). Because striatal MPP^+ levels correlate significantly with the degree of DA neurotoxicity, we also monitored striatal MPP^+ levels in WT versus $PARP^{-/-}$ mice. Previously we had shown that striatal MPP^+ content reaches a peak level approximately 90 min after MPTP injection (15). Accordingly, we determined striatal MPP^+ content at 90 min after four injections of MPTP as well as 2 and 4 hr after the fourth MPTP injection (Table 2). At no time point was the striatal MPP^+ level significantly different between WT and $PARP^{-/-}$ mice. Thus, the absence of the functional PARP protein by genetic knockout accounts for MPTP resistance in $PARP^{-/-}$ mice.

Poly(ADP-ribose) formation via nuclear protein modification is a marker of PARP catalytic activity (20–22, 24, 25, 29, 30, 47–49). In preliminary experiments we examined several time points after MPTP injection to determine when PARP is activated (data not shown). We monitored poly(ADP-ribosyl)ation by using a highly selective and specific mAb to poly(ADP-ribose). We failed to observe any PARP activity in the mouse ventral midbrain, which contains the SNpc until after the fourth injection of MPTP (Fig. 3). Poly(ADP-ribosyl)ation of nuclear proteins is maximal at 2 hr after the fourth injection, and it is still present at 72 hr after the fourth injection. No poly(ADP-ribose) is detected in the striatum of WT animals after MPTP administration, confirming the specificity of the detection of poly(ADP-ribose) (data not shown). In addition to undergoing automodification, PARP catalyzes the poly(ADP-ribosyl)ation of several nuclear proteins, including histones, topoisomerase I and II, DNA polymerase a, proliferating cell nuclear antigen, and p53, all of which play a role in reactions involving DNA strand breaks. PARP is usually the major protein that is poly(ADP-ribosyl)ated; however, unexpectedly we observe that several of these potential alternative acceptors are strongly poly(ADP-ribosyl)ated after MPTP. The precise identities of the ADP-ribosylated proteins under these conditions have not been established; however, based on prior observations, the bands at 116 kDa, 100 kDa, and 53 kDa may represent PARP, topoisomerase I and p53, respectively (49–51). In striking contrast there is no poly(ADP-ribose) formation in the $PARP^{-/-}$ mice after MPTP administration (Fig. 3).

NO is thought to play a major role in activating PARP through its ability to promote nonapoptotic DNA damage (32, 33). To determine whether there is a link between NO and PARP in MPTP-induced parkinsonism, we monitored poly-(ADP-ribosyl)ation after MPTP administration in mice lacking the gene for neuronal NO synthase ($nNOS^{-/-}$) (52). Remarkably, we do not detect any poly(ADP-ribose) formation in $nNOS^{-/-}$ mice (Fig. 3). These data coupled with the observation that NO plays a role in MPTP-induced cell death (12, 13, 15, 16) indicate that NO-induced DNA damage is necessary for PARP activation in MPTP neurotoxicity.

To ascertain whether poly(ADP-ribose) formation was specific to MPTP-injured DA neurons in the SNpc, we monitored the cellular localization of poly(ADP-ribose) formation via immunocytochemistry in the SNpc of WT versus $PARP^{-/-}$ mice and $nNOS^{-/-}$ mice (Fig. 4). We observe intense nuclear poly(ADP-ribosyl)ation in TH-positive SNpc neurons from WT mice but fail to observe any poly(ADP-ribose) formation in nuclei of $PARP^{-/-}$ mice and $nNOS^{-/-}$ mice (Fig. 4).

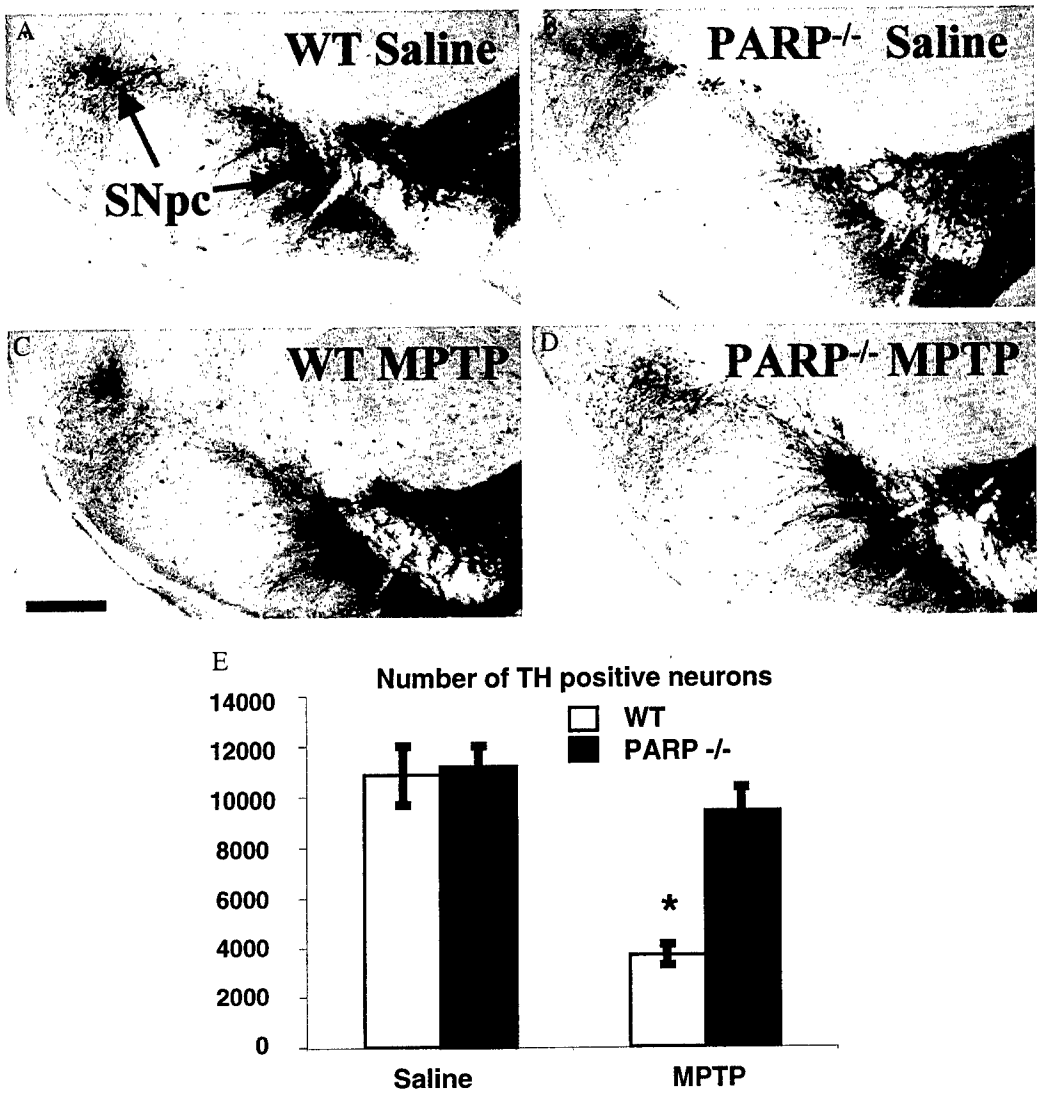

FIG. 2. DA neurons from *PARP*$^{-/-}$ mice are resistant to MPTP neurotoxicity. TH immunostaining of representative midbrain sections 7 days after MPTP adminstration from (*A*) saline-injected WT, (*B*) saline-injected *PARP*$^{-/-}$, (*C*) MPTP-injected WT, and (*D*) MPTP-injected *PARP*$^{-/-}$ mice. (*E*) A significant reduction of TH-immunopositive neurons is seen in the WT mice receiving MPTP ($n = 5$) compared with saline controls ($n = 8$) (*, ANOVA with Fisher post hoc, $P < 0.0001$ WT MPTP vs. saline). No statistical difference is seen between saline controls and *PARP*$^{-/-}$ ($n = 4$) 1 week after MPTP administration (ANOVA). Counts of Nissl-stained neurons in midbrain yielded similar results (data not shown).

DISCUSSION

The major finding of this study is that PARP activation is a principal determinant of MPTP-induced dopaminergic cell death. The profound protection against MPTP neurotoxicity in the *PARP*$^{-/-}$ mice coupled with the observation that DNA is fragmented after MPTP both *in vivo* and *in vitro* (53, 54) implicates DNA damage in MPTP-induced neuronal killing. Previous studies indicate that neuronally derived NO and O_2^- play an important role in MPTP neurotoxicity. Protection against MPTP neurotoxicity is provided by selective nNOS inhibitors or the absence of the nNOS gene (12, 13, 15). MPP^+ directly inhibits complex I of the mitochondria, leading to the generation of superoxide anion (55, 56). The combination of NO and O_2^-, which forms peroxynitrite, is probably the force behind DNA damage in MPTP-induced neurotoxicity as the footprints of peroxynitrite-, nitrotyrosine- (12), and nitrotyrosine-modified proteins (57), are readily detected after MPTP administration. Our observations that PARP is not activated in *nNOS*$^{-/-}$ mice after MPTP links NO, peroxynitrite, and PARP activation in cell death mediated by MPTP. PARP activation is not seen until the fourth injection of MPTP in our paradigm. It is likely that a critical threshold of peroxynitrite formation is required to initiate the cascade of

Table 1. MAO-B catalytic activity in drug naive WT and *PARP*$^{-/-}$ mice

	MAO-B activity	
	K_m, mM	V_{max}, pmol/mg per min
WT	5.21 ± 1.36	148.2 ± 11.9
PARP$^{-/-}$	5.46 ± 1.71	169.7 ± 16.4

Data are the means ± SEM, $n = 3$ for each value. No significant difference exists in MAO-B catalytic activity (ANOVA) between *PARP*$^{-/-}$ or WT.

Table 2. Striatal MPP^+ levels in WT and *PARP*$^{-/-}$ mice

	MPP^+ levels, μg/gm striatum		
	90 min	120 min	240 min
WT	3.33 ± 0.35	1.95 ± 0.38	0.44 ± 0.06
PARP$^{-/-}$	3.03 ± 0.35	2.12 ± 0.28	0.40 ± 0.13

MPP^+ levels were determined at the times indicated after treatment with MPTP. Data are the means ± SEM, $n = 3$ for each value. No significant difference exists in MPP^+ levels (two-way ANOVA) between *PARP*$^{-/-}$ or WT.

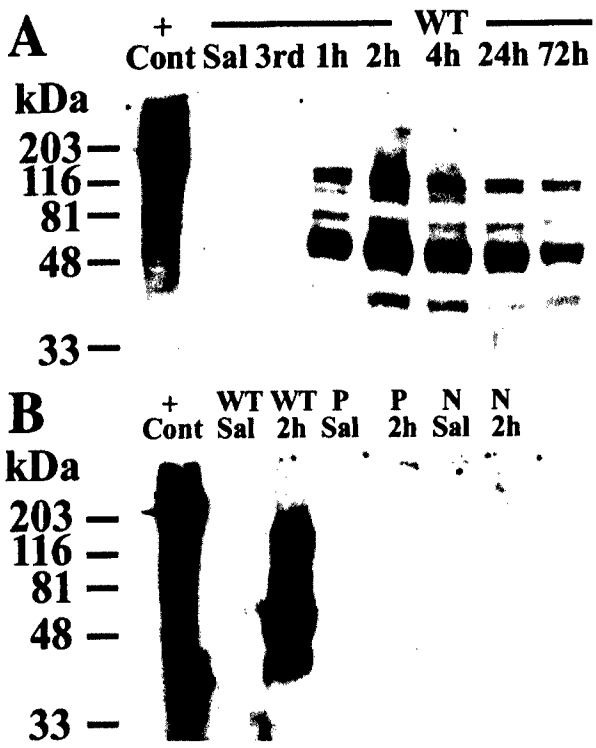

FIG. 3. MPTP induces marked levels of poly(ADP-ribosyl)ation of nuclear proteins. Immunoblot analysis with mAb to poly(ADP-ribose). (*A*) No evidence of poly(ADP-ribosyl)ation is seen until after the fourth injection of MPTP. Polymer formation peaked at 2 hr after the fourth dose and is still detectable at 72 hr. (*B*) No polymer formation is seen in *PARP*$^{-/-}$ or *nNOS*$^{-/-}$ mice. No polymer formation is seen in the striata of WT animals after MPTP (not shown). P, *PARP*$^{-/-}$; N, *nNOS*$^{-/-}$; 3rd, time at the third injection of MPTP; h, hours after the fourth dose of MPTP; +Cont, sonicated Hela cells plus NAD as a positive control. This experiment has been replicated three times with similar results.

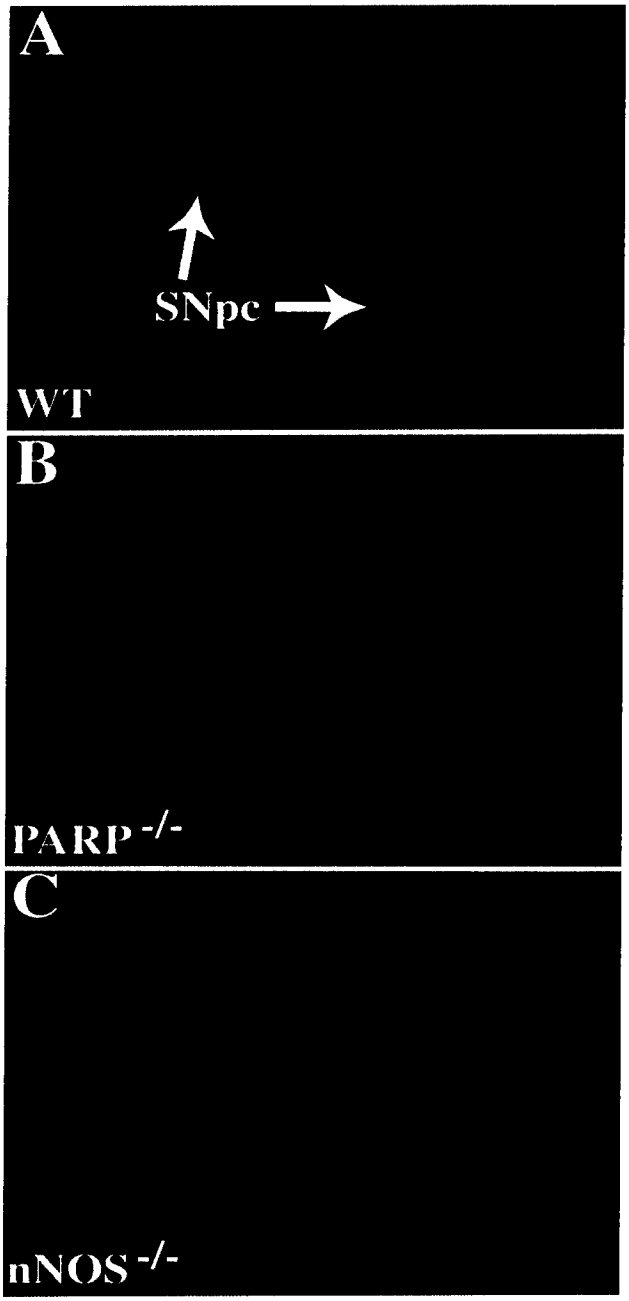

FIG. 4. PARP is activated in DA neurons after MPTP intoxication. Immunohistochemical staining with an anti-poly(ADP-ribose) antibody (pseudocolored in red) in the ventral midbrain. (*A*) WT after MPTP delivery demonstrates intense and specific staining of DA neurons. (*B*) *PARP*$^{-/-}$ midbrains are devoid of immunostaining. (*C*) *nNOS*$^{-/-}$ mice lack poly(ADP-ribose) formation after MPTP. Poly(ADP-ribose) is not detectable in saline-injected animals (data not shown). These images were obtained from animals 4 hr after the fourth injection of MPTP. These experiments have been replicated three times with similar results.

DNA damage and PARP activation. Consistent with this notion are the observations that neuronal death begins after the fourth dose of MPTP (36), coinciding with the peak of peroxynitrite-mediated nitration of TH (57), which parallels the peak of poly(ADP-ribose) formation.

The TH-positive and Nissl-stained neurons of the SNpc are completely spared from the neurotoxic effects of MPTP in the *PARP*$^{-/-}$ mice. This finding contrasts with only a partial, but significant, sparing of striatal DA content in the *PARP*$^{-/-}$ mice. It is well known that the destruction of striatal DA terminals does not necessarily equate with cell loss (58). The complete sparing of dopaminergic neurons, but the loss of striatal DA content may be caused by peroxynitrite-mediated nitration and inactivation of TH (57), the rate-limiting enzyme of DA biosynthesis.

After MPTP neurotoxicity, the following sequence of events presumably takes place. MPTP is converted by MAO-B to MPP^+, which then is taken up into DA neurons via the DA transporter into cell bodies and projections, followed by transport into the mitochondria (42–46). Once there, MPP^+ potently inhibits complex I, which poisons the mitochondrial electron transport chain, leading to decrements in cellular ATP and formation of O_2^-. NO combines with O_2^- to form peroxynitrite, which leads to DNA damage that activates PARP. PARP activation depletes NAD via poly(ADP-ribosyl)ation of nuclear proteins, and ATP is further depleted in an effort to resynthesize NAD, leading to cell death by energy depletion. Consistent with this notion is the observation that replacement of cellular energy stores provides protection against MPTP neurotoxicity (59–61). Although energy depletion is thought to play a prominent role in PARP-mediated cell death (20–22), the pattern of poly(ADP-ribosyl)ation of several nuclear proteins raises the interesting possibility that PARP activation also could contribute to cell death through alternative pathways (20–22, 50, 51). These alternative pathways might account for the profound neuroprotection afforded by deletion of the PARP gene, but only partial protection with replacement of energy stores (59–61).

The role of PARP activation in other forms of cell death, such as cerebral ischemia, glutamate excitotoxicity, cytokine, and free radical-mediated damage to pancreatic islet cells as well as cardiac damage after occlusion of coronary arteries,

suggests that PARP may be a critical choke point in a variety of important pathologic conditions (20–22). The failure to detect PARP activation in $nNOS^{-/-}$ mice after MPTP administration suggests that NO is critical in this process. Although prior studies indicate that the absence of PARP leads to short-term protection against acute injury (20–22), we demonstrate that neurons are alive and functioning for an extended period after a toxic insult. Further understanding of these events may inspire novel therapeutics in the treatment of neurodegenerative disorders.

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Inducible Nitric Oxide Synthase Up-Regulation in a Transgenic Mouse Model of Familial Amyotrophic Lateral Sclerosis

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Abstract: Mutations in copper/zinc superoxide dismutase (SOD1) are associated with a familial form of amyotrophic lateral sclerosis (ALS), and their expression in transgenic mice produces an ALS-like syndrome. Here we show that, during the course of the disease, the spinal cord of transgenic mice expressing mutant SOD1 (mSOD1) is the site not only of a progressive loss of motor neurons, but also of a dramatic gliosis characterized by reactive astrocytes and activated microglial cells. These changes are absent from the spinal cord of age-matched transgenic mice expressing normal SOD1 and of wild-type mice. We also demonstrate that, during the course of the disease, the expression of inducible nitric oxide synthase (iNOS) increases. In both early symptomatic and end-stage transgenic mSOD1 mice, numerous cells with the appearance of glial cells are strongly iNOS-immunoreactive. In addition, iNOS mRNA level and catalytic activity are increased significantly in the spinal cord of these transgenic mSOD1 mice. None of these alterations are seen in the cerebellum of these animals, a region unaffected by mSOD1. Similarly, no up-regulation of iNOS is detected in the spinal cord of age-matched transgenic mice expressing normal SOD1 or of wild-type mice. The time course of the spinal cord gliosis and iNOS up-regulation parallels that of motor neuronal loss in transgenic mSOD1 mice. Neuronal nitric oxide synthase expression is only seen in neurons in the spinal cord of transgenic mSOD1 mice, regardless of the stage of the disease, and of age-matched transgenic mice expressing normal SOD1 and wild-type mice. Collectively, these data suggest that the observed alterations do not initiate the death of motor neurons, but may contribute to the propagation of the neurodegenerative process. Furthermore, the up-regulation of iNOS, which in turn may stimulate the production of nitric oxide, provides further support to the presumed deleterious role of nitric oxide in the pathogenesis of ALS. This observation also suggests that iNOS may represent a valuable target for the development of new therapeutic avenues for ALS. **Key Words:** Superoxide dismutase—Astrocyte—Microglia—Nitric oxide—Inducible nitric oxide synthase—Neuronal nitric oxide synthase—Oxidative stress—Motor neurons—Transgenic mouse—Amyotrophic lateral sclerosis.
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Amyotrophic lateral sclerosis (ALS) is the most frequent neuromuscular disorder in adults and is characterized mainly by progressive muscle wasting and weakness (Rowland, 1995). Mutations in the free radical scavenging enzyme copper/zinc superoxide dismutase (SOD1) gene are associated with a familial form of ALS (Brown, 1995) that is clinically and pathologically indistinguishable from the most common sporadic form of this fatal neurodegenerative disorder. Moreover, transgenic mice that express mutant SOD1 (mSOD1) develop an adult-onset paralytic condition that reproduces the clinical and pathological hallmarks of ALS (Gurney et al., 1994; Ripps et al., 1995; Wong et al., 1995). We (Kostic et al., 1997*b*) and others (Chiu et al., 1995; Dal Canto and Gurney, 1995) have found that the level of mSOD1 expression markedly modulates the age of onset of symptoms, but has no or minimal effect on the type of symptoms or the rate of progression of the disease. This suggests that, although mSOD1 is a pivotal factor in the initiation of motor neuron disease, additional factors contribute to the propagation of the neurodegenerative process. To elucidate such factors, and consequently to develop new therapies aimed at stopping or slowing the progression of ALS, we revisited its neuropathology in search of abnormalities that could shed light on these putative culprits. Aside from the dramatic loss of motor neurons, which predominates in the anterior horn, it is worth mentioning that the gray matter of the spinal cord is also the site of a robust glial reaction in both humans and transgenic mice (Adams et al., 1984; Hirano, 1996; Schiffer et al., 1996; Hall et al., 1998). Although gliosis may, in certain situations, mediate beneficial phenomena,

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Abbreviations used: ALS, amyotrophic lateral sclerosis; GFAP, glial fibrillary acidic protein; iNOS, inducible nitric oxide synthase; mSOD1, mutant copper/zinc superoxide dismutase; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; SDS, sodium dodecyl sulfate; SOD1, copper/zinc superoxide dismutase; TBS, Tris-buffered saline; wtSOD1, wild-type copper/zinc superoxide dismutase.

a greater number of situations come to mind in which gliosis may be implicated in harmful events. Accordingly, the purpose of the study was to characterize concomitantly the temporal and topographic distribution of the reactive astrocytes and activated microglial cells and of the nitric oxide (NO) synthesizing isoenzyme inducible NO synthase (iNOS) in the spinal cord of transgenic mSOD1 mice. Supporting the choice to study iNOS is the presumed pathogenic role of NO in ALS (Beckman et al., 1993; Beal et al., 1997; Ferrante et al., 1997) and the central role of iNOS in NO production by reactive astrocytes and/or activated microglial cells (Nathan and Xie, 1994*a*).

To achieve the stated goal, we studied the protein expression of the astrocytic marker glial fibrillary acidic protein (GFAP), of the microglial marker MAC-1, and of iNOS in the spinal cord (the prototypic region affected by mSOD1) and the cerebellum (the prototypic region unaffected by mSOD1) of asymptomatic, early symptomatic, and end-stage transgenic mice expressing the G93A mutant of SOD1; these mice have been extensively validated (Gurney et al., 1994; Chiu et al., 1995; Dal Canto and Gurney, 1995; Kostic et al., 1997*a*,*b*) and express a SOD1 point mutation that causes ALS in humans (Brown, 1995). The catalytic activity of iNOS and the level of mRNA in the spinal cord of these transgenic mice were also determined, at the three selected disease stages.

In the brain, NO can also be produced by another NO synthase (NOS) isoform, neuronal NOS (nNOS), which is believed to be a constitutive enzyme exclusively expressed by neurons (Dawson and Snyder, 1994). However, disputing this view is the recent and unexpected finding that nNOS is expressed not only in neurons, but also in reactive astrocytes at the level of the spinal cord in transgenic mSOD1 mice (Cha et al., 1998). This observation prompted us also to assess nNOS protein expression and catalytic activity in the spinal cord of the transgenic mSOD1 mice at the same three selected disease stages.

EXPERIMENTAL PROCEDURES

Reagents

All general laboratory reagents were from Sigma (St. Louis, MO, U.S.A.) unless stated otherwise and were of the highest purity grade. The monoclonal anti-GFAP antibody was from Boehringer Mannheim (Indianapolis, IN, U.S.A.), the monoclonal anti-MAC-1 antibody was from Serotec (Raleigh, NC, U.S.A.), the polyclonal anti-nNOS antibody was from Zymed (San Francisco, CA, U.S.A.), and the polyclonal anti-iNOS antibody was from Transduction Laboratories (Lexington, KY, U.S.A.). All secondary antibodies and normal sera were from Vector (Burlingame, CA, U.S.A.). Pairs of primers for iNOS and β-actin were obtained from GibcoBRL (Grand Island, NY, U.S.A.).

Animals

Hemizygote transgenic mSOD1 mice and their wild-type littermates were used in this study. This transgenic mouse line (G1H) carries the point mutation Gly $\rightarrow$ Ala at codon 93 of the human SOD1 gene and expresses ~18 copies of human mutated gene (Gurney et al., 1994). To assess the effect of overexpression of the wild-type human SOD1 enzyme (wtSOD1), hemizygote transgenic wtSOD1 mice (Jackson Laboratory, Bar Harbor, ME, U.S.A.) were used. This transgenic mouse line (N29) expresses >10 copies of human gene (Gurney et al., 1994). In preliminary studies, we measured SOD1 catalytic activity in spinal cord homogenates of 6-week-old transgenic mSOD1 (G1H) and transgenic wtSOD1 (N29) mice and found that both mouse lines have greater than sixfold higher SOD1 activity as compared with their wild-type littermates; SOD1 activity was determined according to the method described previously (Przedborski et al., 1996*a*; Kostic et al., 1997*a*).

On postnatal day 14, 20 μl of blood from the tail was collected from each mouse pup and used for SOD1 activity gel electrophoresis (Przedborski et al., 1992), and the piece of clipped tail was used for PCR as described (Kostic et al., 1997*b*) using a pair of primer that is specific for exon-4 of human SOD1.

After being genotyped, animals were divided into transgenic mSOD1 mice, transgenic wtSOD1 mice, and wild-type mice (i.e., nontransgenic littermates from transgenic mSOD1 mice). All studies were performed on three to six mice per group and at three different clinical stages of the transgenic mSOD1 mice as defined (Kostic et al., 1997*a*): asymptomatic (~6 weeks old), early symptomatic [117 $\pm$ 6 days of age (mean $\pm$ SD)], and end-stage (165 $\pm$ 2 days of age). Their nontransgenic mSOD1 littermates were killed at the same time. To have age-matched control mice expressing wild-type SOD1, transgenic wtSOD1 mice were killed at 168 days of age. Until being killed, nontransgenic mSOD1 and transgenic wtSOD1 mice did not show any behavioral abnormalities. This experimental protocol was approved by the Animal Care and Use Committee of Columbia University and is in agreement with the guidelines from the National Institutes of Health for the use of live animals.

Immunohistochemistry

All mice were anesthetized (pentobarbital, 30 mg/kg i.p.) and perfused intracardially with 25 ml of normal saline followed by 75 ml of 4% (wt/vol) paraformaldehyde in 0.1 *M* phosphate buffer (4% PF/PB; pH 7.1). Spinal cord and cerebellum were then dissected out on ice, postfixed by immersion in 4% PF/PB (4 h, 4°C), cryoprotected in 30% (wt/vol) sucrose in phosphate buffer, and frozen by immersion in isopentane cooled on dry ice. Frozen spinal cord and cerebellum samples were cut (30 μm) in a cryostat; from the spinal cord 20–40 serial sections from cervical (C7) and lumbar (L3) levels were collected, whereas from the cerebellum only a few transversal sections were collected. Spinal segments were identified by location of spinal roots and by the characteristic morphology of the spinal cord. For all immunostainings, sections were collected in ice-cold phosphate buffer free floating, and then successively rinsed (3 $\times$ 5 min) in 0.1 *M* Tris-HCl (pH 7.4) buffer containing 9 g/L NaCl (TBS), incubated in 5% normal goat serum in TBS containing 0.1% Triton X-100 (60 min, 25°C), and incubated with one of the primary antibodies [GFAP (1:500); MAC-1 (1:1,000); iNOS (1:1,000); nNOS (1:1,000)] containing 2% normal serum (24–48 h, 4°C). After 3 $\times$ 5 min rinses in TBS, sections were successively incubated (1 h, 25°C) in biotinylated-conjugated polyclonal secondary antibody (1:200), rinsed (3 $\times$ 5 min) in TBS, incubated in horseradish-conjugated avidin/biotin complex (Vector), rinsed (3 $\times$ 5 min) in TBS, and incubated in diaminobenzidine/H_2O_2. Then all sections were mounted on glass slides and counter-

stained with thionin before being dehydrated in alcohol, cleared in xylene, and coverslipped.

Specificity of nNOS and iNOS immunoreactivity was assessed by incubating adjacent spinal cord tissue sections with and without primary antibody. Specificity of nNOS and iNOS was also assessed by western blot analysis using a standard protocol (Ara et al., 1998). For nNOS, mouse cerebellum was dissected, homogenized in 2.5 volumes (wt/vol) of ice-cold 50 m*M* Tris-HCl (pH 7.0) buffer containing 150 m*M* NaCl, 5 m*M* EDTA, 1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, and protease inhibitors (Mini Cocktail; Boehringer Mannheim), and centrifuged (16,000 *g*, 30 min, 4°C). For iNOS, mouse macrophage cells were stimulated with interferon-γ and lipopolysaccharide for 12 h as described (Xie et al., 1992), and cell lysate was processed as described above for cerebellum. For both nNOS and iNOS, supernatant was mixed with an equal volume of 2× sample buffer containing SDS and 2-mercaptoethanol, boiled 5 min, and fractionated by miniature 12% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, U.S.A.) and reacted with either a polyclonal rabbit anti-nNOS (1:1,000 dilution; Zymed) or anti-iNOS antibody (1:1,000; Transduction Laboratories). Bound primary antibody was detected by probing with a horseradish-conjugated polyclonal anti-rabbit antibody (1:2,000 dilution; Amersham). After washing, the nitrocellulose was incubated for 5–10 min with chemiluminescent substrate (SuperSignal Ultra, Pierce Chemical, Rockford, IL, U.S.A.) and then exposed to x-ray film (Kodak BioMax MS) for ~1 min.

Total RNA preparation and RT-PCR

Total RNA from both spinal cord and cerebellum of transgenic mSOD1, transgenic wtSOD1, and wild-type littermates was prepared using RNeasy kit (Qiagen Inc., Valencia, CA, U.S.A.) according to the manufacturer's instructions. The concentration and purity of RNA preparations were determined by measuring the absorbance at 260 and 280 nm in a spectrophotometer. First-strand cDNA was synthesized from total RNA using SuperScript Preamplification System with SuperScript II RNase H-reverse transcriptase (GibcoBRL) according to the manufacturer's instructions. Then 1 μl of cDNA template was amplified by PCR in a 20-μl total reaction volume containing 18 μl of Supermix (GibcoBRL) and 4–10 pmol of each specific primer. The iNOS primer sequences were 5′-TCACTGGGACAGCACAGAAT-3′ (forward) and 5′-TGTGTCTGCAGATGTGCTGA-3′ (reverse). As internal control, β-actin cDNA was coamplified using primer sequences 5′-CTTTGATGTCACGCACGATTTC-3′ (forward) and 5′-GGGCCGCTCTAGGCACCAA-3′ (reverse). Each PCR cycle consisted of 45 s at 94°C, 45 s at 60°C, and 1 min at 72°C. PCR amplification was carried out for 35 cycles for iNOS and for 28 cycles for β-actin; the conditions for each PCR amplification resulted in an exponential amplification range for quantification of each mRNA. After amplification, the products were separated on a 1.5% agarose gel containing 0.03% ethidium bromide. Bands were then visualized under UV illumination, and gels were photographed with Polaroid-665 positive/negative films. Negatives were then digitized, each band was outlined with a screen cursor driven by a hand-held mouse, and optical densities were determined using a computerized image analysis system (Inquiry image analyzer, Loats Associates, Westminster, MD, U.S.A.).

Assay of NOS catalytic activity

Spinal cords of transgenic mSOD1 (asymptomatic, early symptomatic, and end-stage) and of wild-type littermates were quickly removed, immediately frozen on dry ice, and stored at −80°C until analysis. NOS catalytic activity was assayed by measuring both the Ca^{2+}-dependent and the Ca^{2+}-independent conversion of [^{3}H]arginine to [^{3}H]citrulline as described by Przedborski et al. (1996*b*). On the day of the assay, tissue samples were homogenized in 10 volumes (wt/vol) of 50 m*M* Tris-HCl (pH 7.4) buffer containing 1 m*M* EDTA and 1 m*M* EGTA. Then 25 μl of homogenate was added to 100 μl of 50 m*M* Tris-HCl (pH 7.4) buffer containing 1 m*M* NADPH, 1 m*M* EDTA, 1 m*M* EGTA, and 0.1 μCi L-[2,3-^{3}H]arginine (specific activity: 36.8 Ci/mmol; Du Pont–NEN, Boston, MA, U.S.A.) in the presence or absence of 2.25 m*M* $CaCl_2$, and incubated for 15 min at 25°C. The reaction was terminated by the addition of 3 ml of 20 m*M* HEPES (pH 5.5), 1 m*M* EDTA, and 1 m*M* EGTA, and the entire reaction mixture was applied onto a 0.5-ml column of Dowex AG50WX-8 (Pharmacia, Piscataway, NJ, U.S.A.). [^{3}H]Citrulline was quantified by liquid scintillation counting of the eluate. No significant [^{3}H]citrulline production occurred in the absence of NADPH.

Protein and hemoglobin determination

Protein content was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard. Hemoglobin content was determined by quantifying the oxidation of 3,3′,5,5′-tetramethylbenzidine by hydrogen peroxide (Lijana and Williams, 1979).

Statistics

For each experiment, three to six mice per group were studied, and values are expressed as means ± SEM unless stated otherwise. Differences among means were analyzed using one-way ANOVA with the different types of mice as the independent factor. When ANOVA showed significant differences, pairwise comparisons between means were tested by Newman–Keuls post-hoc testing. In all analyses, the null hypothesis was rejected at the 0.05 level. All statistical analyses were performed using SigmaStat for Windows (version 2.0, Jandel Corp., San Rafael, CA, U.S.A.).

RESULTS

Spinal cord astrocytic and microglial reaction in transgenic mSOD1 mice

To assess whether a glial reaction takes place under the deleterious effect of mSOD1 and whether it occurs specifically in affected brain regions, we studied by immunohistochemistry the time course and anatomical distribution of GFAP and MAC-1, two specific markers of astrocytes and microglial cells, respectively. In all types of mice studied, significant GFAP immunoreactivity was seen in the white matter of the spinal cord at both the cervical and lumbar levels. The immunostained structures appeared essentially as fine tortuous processes likely corresponding to resting astrocytes (Fig. 1A). Robust GFAP immunoreactivity was seen in the gray matter of the spinal cord only in end-stage (Fig. 1B) and, to a lesser extent, in early symptomatic transgenic mSOD1 mice. Particularly striking in the end-stage transgenic mSOD1 mice, GFAP immunostaining predominated in the anterior horn of the spinal cord (Fig. 1B and C) and

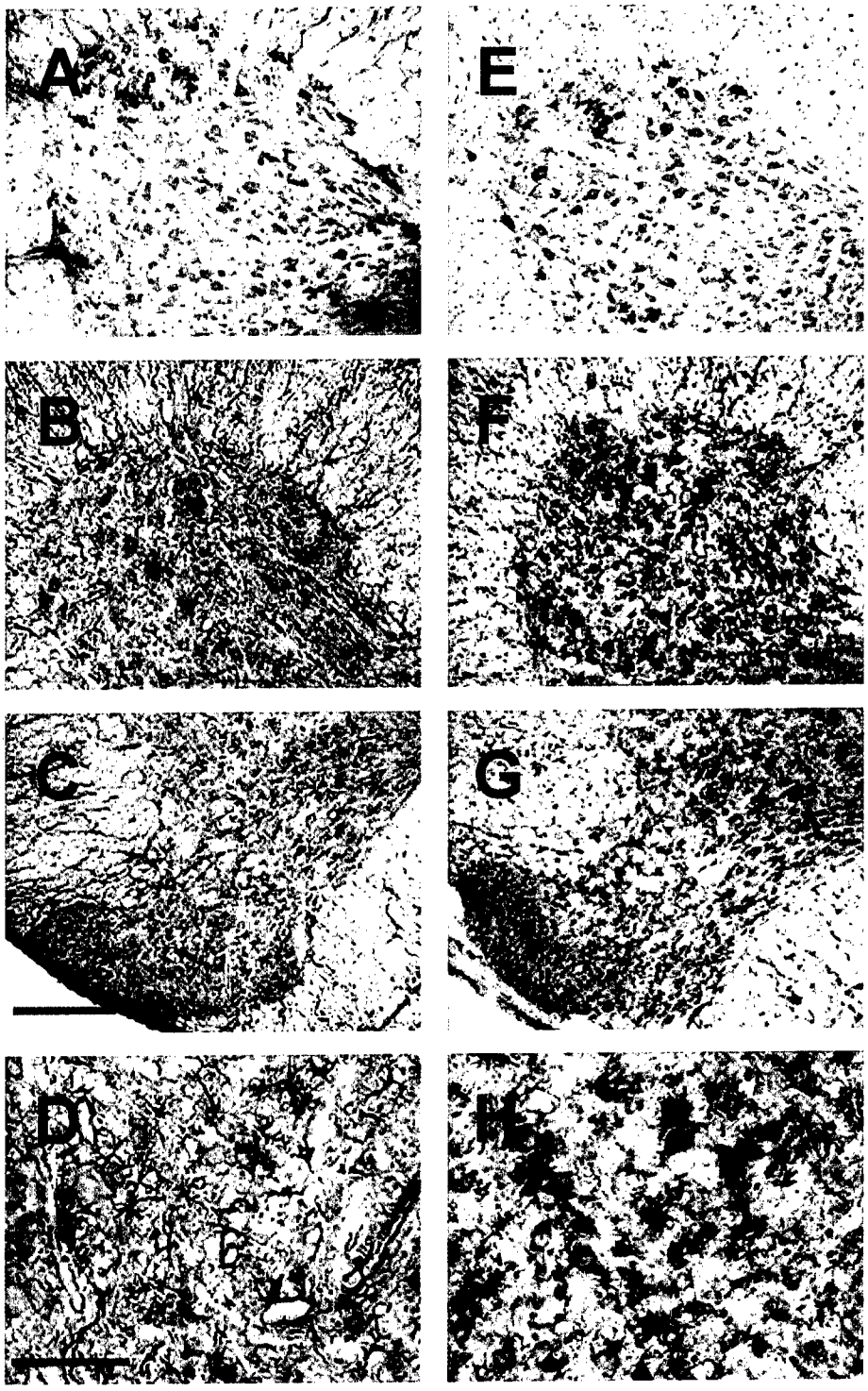

FIG. 1. Representative photomicrographs illustrating the glial and microglial reaction in the spinal cord of an end-stage transgenic mSOD1 mouse. Except for the white matter where significant immunostaining is observed, only negligible GFAP and MAC-1 immunoreactivity is seen in wild-type age-matched control (**A** and **E**). In contrast, strong GFAP and MAC-1 immunoreactivity is detected in the gray matter of the end-stage transgenic mSOD1 mouse (**B** and **F**), which, at higher magnification, corresponds to reactive astrocytes (**D**) and microglial cells (**H**). Also note that the glial reaction is more robust in the anterior horn (B and F) than in the posterior horn (**C** and **G**). Scale bar = 200 μm in A–C and E–G and 50 μm in D and H.

appeared as thick processes and occasional small cell bodies (Fig. 1D), consistent with these being reactive astrocytes; asymptomatic transgenic mSOD1 mice, as well as wild-type and transgenic wtSOD1 mice, showed minimal or mild GFAP immunoreactivity in the gray matter of the spinal cord (Fig. 1A). Likewise, no increase in GFAP immunostaining was seen in unaffected brain regions of end-stage transgenic mSOD1 mice, such as the cerebellum.

Alterations very similar to those described for astrocytes were seen for microglia cells. Indeed, numerous MAC-1-positive fine tortuous processes, likely corresponding to resting microglial cells, were seen in the white matter of the spinal cord from all groups of mice.

In addition, conspicuous MAC-1 immunostaining was seen in the gray matter of the spinal cord of both early symptomatic and end-stage transgenic mSOD1 mice (Fig. 1F). Here, immunostained elements appeared as small cell bodies with thick, short processes (Fig. 1H) consistent with their being activated microglial cells. In several instances, activated microglial cells were grouped around neurons (Fig. 1H), realizing an image of neuronophagy (Adams et al., 1984). An identical finding was observed with another antibody directed against the specific microglial membrane antigen F4/80 (data not shown).

iNOS- and nNOS-positive cells in spinal cord of transgenic mSOD1 mice

To determine whether the strong observed spinal cord gliosis is associated with iNOS up-regulation, we first examined iNOS protein expression in the spinal cord of transgenic mSOD1 mice by immunohistochemistry. No iNOS immunoreactivity was seen in either the white or gray matter of the spinal cord of asymptomatic transgenic mSOD1 mice, transgenic wtSOD1 mice, or wild-type littermates (Fig. 2A). Conversely, iNOS immunostaining was observed in the gray matter and preferentially in the anterior horn of the spinal cord of early symptomatic and end-stage transgenic mSOD1 mice (Fig. 2B and C). Immunolabeling for iNOS appeared slightly more intense in spinal cord sections from cervical compared with lumbar level and from end-stage compared with early symptomatic transgenic mSOD1 mice. Immunostaining for iNOS was only identified in structures reminiscent of glial cells and not of neurons (Fig. 2D). Also, no iNOS immunoreactivity was seen in the cerebellum of end-stage transgenic mSOD1 mice and, in the absence of anti-iNOS antibody, none of the spinal cord iNOS immunostaining described above could be seen.

nNOS protein expression was also studied by immunohistochemistry, but here, inversely to the situation observed for iNOS, nNOS immunostaining decreased over the course of the disease. The most abundant nNOS immunostaining was seen in the gray matter of the spinal cord of wild-type mice (Fig. 2E) with no obvious difference from that seen in asymptomatic transgenic mSOD1 mice and transgenic wtSOD1 mice. In these mice, the most intense immunostaining was seen in neurons in the vicinity of the central canal, whereas several motor neurons in the anterior horn exhibited a much fainter immunolabeling (see inset in Fig. 2E). It was also clear that, in the spinal cord of transgenic mSOD1 mice, the greater the loss of motor neurons, the smaller the number of nNOS-positive neurons (Fig. 2F). Moreover, under the present conditions, no nNOS immunostaining could be detected in cells other than neurons in the spinal cord of early symptomatic or end-stage transgenic mSOD1 mice (Fig. 2G). However, in these mice several spinal cord neurons, as well as their surrounding neuropil, showed more intense nNOS immunoreactivity (Fig. 2F and G) compared with control mice (Fig. 2E). As for iNOS, omission of anti-nNOS antibody was associated with no positive immunostaining in the spinal cord of any of the groups of mice studied (Fig. 2H). Specificity of both nNOS and iNOS antibody was confirmed by western blot analysis (Fig. 3). Immunoblots from whole cerebellar proteins showed a prominent band at ~160 kDa (lane 1), which is consistent with the molecular mass of nNOS, in the presence of the anti-nNOS antibody; this band was not observed when the anti-nNOS antibody was omitted (lane 2). Similarly, immunoblots from stimulated cell lysate showed a single band at ~130 kDa (lane 3), which is consistent with the molecular mass of iNOS, in the presence of the anti-iNOS antibody; this band was not observed when the anti-iNOS antibody was omitted (lane 4).

Up-regulation of iNOS mRNA in transgenic mSOD1 mice

To determine whether augmentation in iNOS protein expression is accompanied by changes in iNOS mRNA, we tested spinal cord samples from the different groups of mice by RT-PCR. Consistent with the findings for iNOS protein, iNOS mRNA expression also differed among the different groups of mice [$F(3, 8) = 7.8$, $p < 0.009$] (Fig. 4). In spinal cord from early symptomatic transgenic mSOD1 mice, iNOS mRNA appeared to be about three times more abundant than that in spinal cord from wild-type mice (Fig. 4). Similarly, in spinal cord from asymptomatic and end-stage transgenic mSOD1 mice, iNOS mRNA appeared also to be more abundant than that in spinal cord from wild-type mice (Fig. 4), but here the difference was less impressive and did not reach significance. To control for the specificity of the PCR reaction, we also performed the reaction using normal mouse genomic DNA and mRNA subjected to cDNA synthesis without RT; neither reaction produced iNOS PCR products.

It is important to point out that all tissue samples used for RT-PCR were not perfused and, hence, contained some blood. However, the amount of blood trapped in the spinal cord tissue samples, determined by measuring the content of hemoglobin, was comparable [$F(3, 8) = 0.012$, $p = 0.99$] among wild-type (4.4 ± 0.7 μg/mg of protein), asymptomatic transgenic mSOD1 (4.8 ± 0.5 μg/mg of protein), early symptomatic transgenic mSOD1 (4.3 ± 2.6 μg/mg of protein), and end-stage transgenic mSOD1 mice (4.5 ± 2.8 μg/mg of protein). Accordingly, it is unlikely that the differences in iNOS mRNA observed by RT-PCR among the different groups of samples could be due to different amounts of blood-borne iNOS mRNA.

Alterations of nNOS and iNOS activity in transgenic mSOD1 mice

To demonstrate whether the observed changes in nNOS and iNOS protein are associated with detectable alterations in catalytic activity, we measured the conversion of [^{3}H]arginine into [^{3}H]citrulline in spinal cord homogenates from the different groups of mice. In the

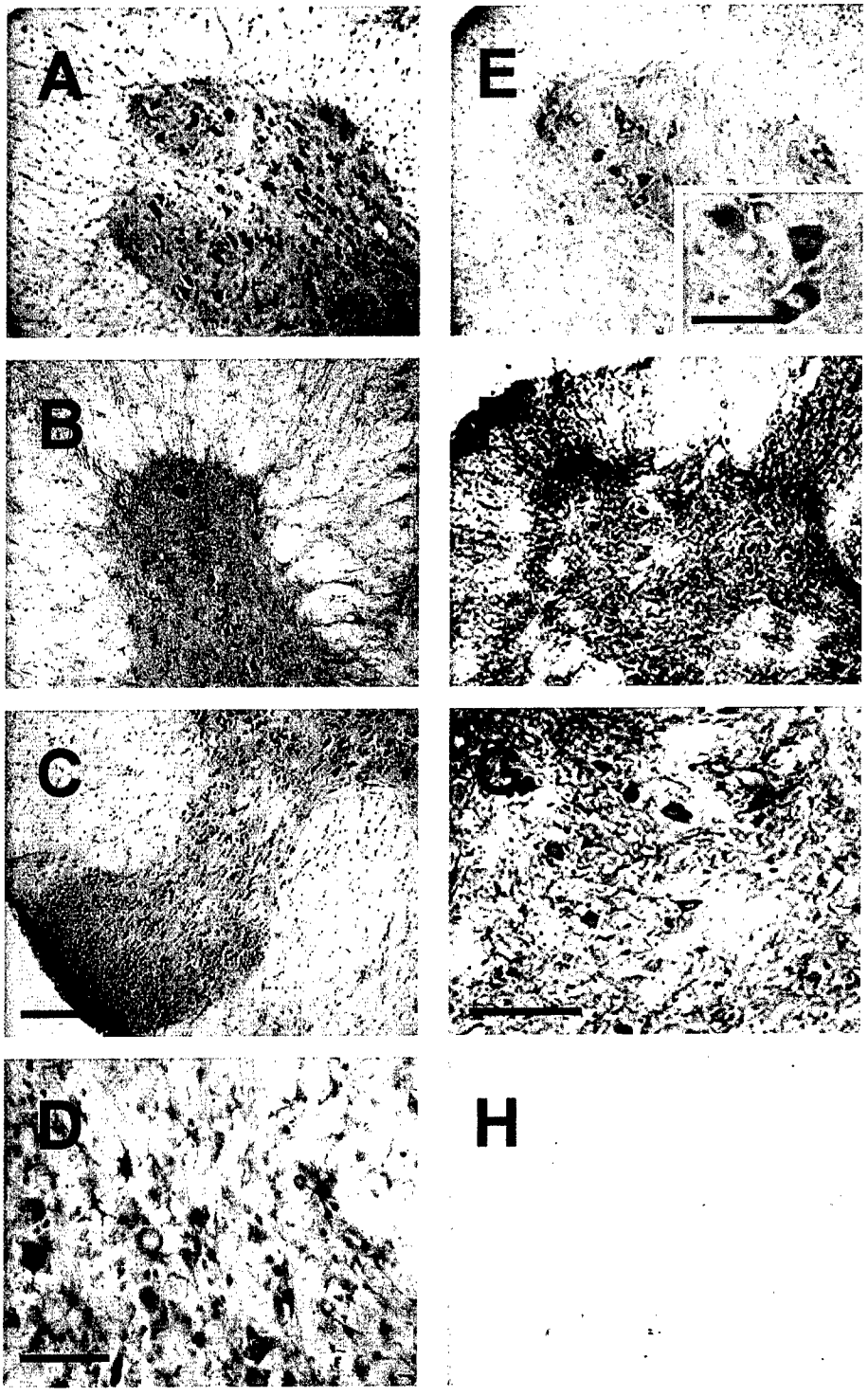

FIG. 2. Representative photomicrographs illustrating the changes in iNOS and nNOS in the spinal cord of an end-stage transgenic mSOD1 mouse. No detectable iNOS immunoreactivity is observed in wild-type age-matched control (**A**), whereas nNOS immunoreactivity is observed in a few scattered cells (**E**) with the morphology of healthy neurons (see **inset**). Strong spider-like iNOS immunoreactivity is seen throughout the spinal cord of end-stage transgenic mSOD1 mouse (**B**), which, at higher magnification, seems to correspond to glial cells (**D**). iNOS immunoreactivity is more abundant in the anterior horn (B) than in the posterior horn (**C**). Of note, in the end-stage transgenic mouse the number of nNOS-positive neurons is smaller (**F**) and no nNOS immunoreactivity is identified in nonneuronal elements (E and **G**). In the absence of iNOS or nNOS antibody, no specific immunoreactivity is seen (**H**). Scale bar = 200 μm in A–C, E, F, and H and 50 μm in D and G.

presence of Ca^{2+} the conversion of [^{3}H]arginine, which essentially reflects nNOS catalytic activity, decreased significantly over the course of the degenerative process [$F(3, 8) = 8.2$, $p < 0.008$]. For instance, spinal cord nNOS activity was >65% lower in end-stage transgenic mSOD1 mice compared with age-matched wild-type controls (Fig. 5A). In contrast, in the absence of Ca^{2+} the conversion of [^{3}H]arginine, which essentially reflects iNOS catalytic activity, increased significantly over the course of the degenerative process [$F(3, 8) = 5.8$, $p < 0.021$]. Here, iNOS activity is about seven times higher in early symptomatic and end-stage transgenic mSOD1 mice compared with wild-type controls (Fig. 5B).

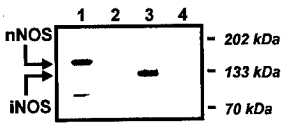

FIG. 3. nNOS and iNOS western blot. Lane 1 (20 μg of mouse cerebellar proteins immunostained with the nNOS antibody) shows a prominent band at 160 kDa that corresponds to nNOS; when the antibody is omitted, the band is not seen (lane 2). Lane 3 (7 μg of stimulated mouse cell lysate immunostained with the iNOS antibody) shows a single band at 130 kDa that corresponds to iNOS; when the antibody is omitted, the band is not seen (lane 4).

DISCUSSION

The present study shows that, aside from the dramatic loss of motor neurons, gliosis is, as in human ALS, a striking neuropathological feature of the spinal cord of transgenic mSOD1 mice (Adams et al., 1984; Hirano, 1996; Schiffer et al., 1996; Hall et al., 1998). Our data on gliosis in these mice emphasize three points: first, it appears specific to affected regions, because cerebellum, a brain region devoid of neuropathological changes in this model (Dal Canto and Gurney, 1995), did not show any evidence of gliosis; second, it is related to the cytotoxic effect of the mutant protein and not to increased SOD1 activity, because age-matched transgenic mice with increased wild-type SOD1 activity did not develop

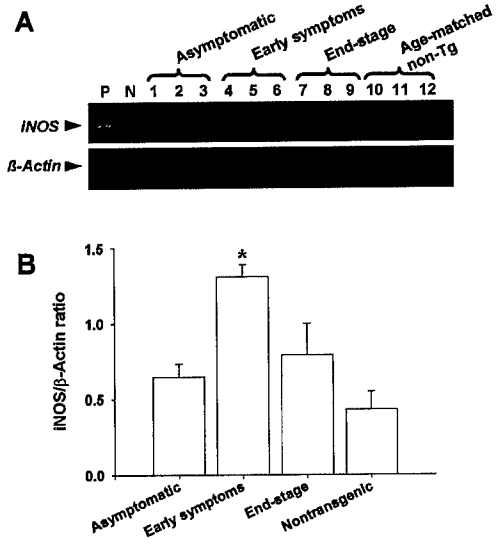

FIG. 4. Spinal cord iNOS RT-PCR. Photograph of the gel (**A**) and the corresponding bar graph (**B**) show significantly more iNOS mRNA in early symptomatic and end-stage transgenic mSOD1 mice compared with asymptomatic mSOD1 mice and wild-type age-matched controls. Experiments were performed as described in Experimental Procedures. The presented data are from three mice per group and are representative of at least two independent experiments. P, iNOS positive control (murine cells treated with lipopolysaccharide); N, negative control (product of cDNA synthesis in absence of RT); non-Tg, nontransgenic. $^{*}p < 0.05$ (Newman–Keuls post-hoc) compared with all other groups.

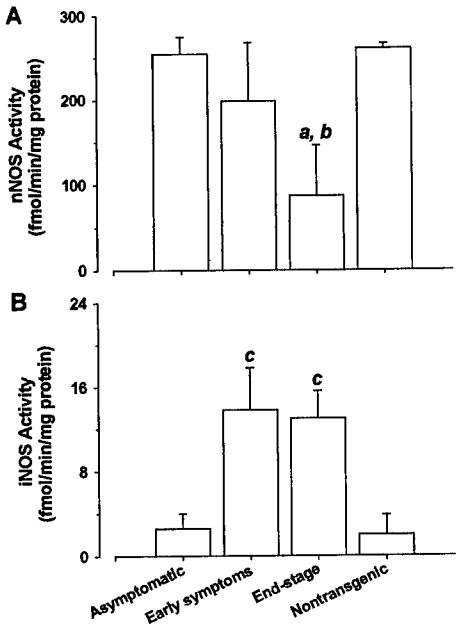

FIG. 5. Spinal cord nNOS and iNOS activities. nNOS activity (**A**), as assessed by the conversion of [^{3}H]arginine to [^{3}H]citrulline in the presence of Ca^{2+}, significantly decreases over the course of the disease. Conversely, iNOS activity (**B**), as assessed by the conversion of [^{3}H]arginine to [^{3}H]citrulline in the absence of Ca^{2+}, is significantly increased in early symptomatic and end-stage transgenic mSOD1 mice compared with asymptomatic transgenic mSOD1 and wild-type mice. Assays were performed in duplicate as described in Experimental Procedures in three mice per group. $^{a}p < 0.01$ (Newman–Keuls post-hoc) compared with asymptomatic transgenic mSOD1 and wild-type mice; $^{b}p < 0.05$ (Newman–Keuls post-hoc) compared with early asymptomatic transgenic mSOD1; $^{c}p < 0.05$ (Newman–Keuls post-hoc) compared with asymptomatic transgenic mSOD1 and wild-type mice.

gliosis; and third, it closely parallels the time course of motor neuron loss, because no evidence of gliosis was observed before any actual motor neuron loss (e.g., 6-week-old transgenic mice). We also found that both astrocytes and microglial cells reacted in concert (Fig. 1). However, if we failed to see any obvious quantitative differences between the reactive astrocytes and activated microglial cells, we clearly saw qualitative differences (Fig. 1). For instance, reactive astrocytes in the anterior horn were present throughout the gray matter of early symptomatic and, even more so, of end-stage transgenic mSOD1 mice without showing any preferential distribution within the damaged tissue (Fig. 1A and B). In contrast, not only were activated microglial cells, like reactive astrocytes, diffusely distributed throughout the anterior horn, but in several discrete areas of the damaged gray matter they formed a typical image of neuronophagy (Fig. 1D and H). This neuropathological feature is common to all forms of encephalitis that affect the gray matter, and is often present in ALS, especially in cases with rapid progression (Adams et al., 1984). Although the exact mechanism underlying neuronophagy is unknown, the belief is that it results from the release of

chemotactic substances by dying neurons, which, in turn, attract microglial cells. Collectively, these findings suggest that the observed gliosis is related to the neurodegenerative process and is most likely secondary to the loss of motor neurons, rather than a primary event. Although this view undercuts the potential role of gliosis in initiating the disease, it does not undermine the potential role of gliosis in propagating the disease. Consistent with this is the observed gliosis-related up-regulation of iNOS, an enzyme that produces high amounts of NO for a prolonged period of time (Nathan and Xie, 1994*b*), as well as superoxide radicals (Xia and Zweier, 1997), two reactive species that can either directly or indirectly promote neuronal death.

In normal situations, iNOS is not expressed by glial cells, but is in pathological conditions of the CNS, including stroke (Iadecola et al., 1995), HIV infection (Adamson et al., 1996), multiple sclerosis (Bagasra et al., 1995), Alzheimer's disease (Vodovotz et al., 1996), and Parkinson's disease (Hunot et al., 1996). The present study suggests that ALS may well be an additional neurological disease in which iNOS plays a role, as we found that iNOS was up-regulated in glial cells of the spinal cord in early symptomatic and end-stage transgenic mSOD1 mice (Fig. 2A and B). In this model of ALS, as for gliosis, iNOS expression seemed to parallel spinal cord neurodegeneration and not to precede it, and was not observed in the cerebellum or in any studied regions of the CNS of age-matched transgenic wtSOD1 and wild-type mice. We also have the proof that the increased iNOS immunoreactivity seen in the spinal cord of the transgenic mSOD1 mice corresponds to the expression of a catalytically active form of the protein, because the Ca^{2+}-independent conversion of [^{3}H]arginine into [^{3}H]citrulline was increased significantly in the spinal cord of these animals compared with age-matched controls (Fig. 5). This latter finding not only strengthened the veracity of the immunostaining data, but also, and more importantly, provided meaningful functional information that allows one to believe that iNOS up-regulation in this model of ALS may have practical repercussions on the neurodegenerative process.

Both in vitro and in vivo experiments have demonstrated that iNOS transcription can be induced in astrocytes and/or microglial cells by bacterial lipopolysaccharide, as well as by various cytokines, including tumor necrosis factor-α, interleukin-1β, and interferon-γ (Galea et al., 1992; Murphy et al., 1993; Garcion et al., 1998). In light of this, it is particularly relevant to ALS to mention that inhibition of interleukin-1β activation prolongs survival of transgenic mSOD1 mice (Friedlander et al., 1997) and that tumor necrosis factor-α levels are increased in the spinal cord of the *mnd* mouse (Ghezzi et al., 1998), which represents another experimental model of motor neuron disease. In agreement with this transcriptional induction model of iNOS, we demonstrated that iNOS mRNA levels are increased in the spinal cord of early symptomatic and end-stage transgenic mSOD1 mice (Fig. 4). Our data are therefore consistent with the current knowledge of iNOS regulation and suggest the following scenario. Subsequent to the initiation of the disease, motor neurons die, which causes the release of various compounds, including chemotactic factors and cytokines, from the neurons themselves and/or from other cellular sources. This leads to the formation of gliosis and the induction of iNOS, which in turn, as indicated above, stimulates the formation of $\dot{N}O$ and other reactive species, hence subjecting remaining motor neurons to an oxidative stress.

Only neurons were labeled with the anti-nNOS antibody used in the present study. nNOS-positive neurons were scattered throughout the spinal cord, and among those seen in the anterior horn only a few had a morphology of large motor neurons (Fig. 2E). Consistently, nNOS-positive neurons located near the central canal were heavily labeled, whereas those in the ventral and dorsal horns were labeled much less intensively (see inset in Fig. 2E), although among nNOS-positive motor neurons the intensity of the labeling was highly variable. This description is in agreement with that reported for several animal species (Dun et al., 1993). Particularly noticeable in end-stage transgenic mSOD1 mice is the reduction of nNOS-positive neurons, especially at the level of the anterior horn (Fig. 2F). Although no formal cell counts have been made, it appears that the loss of nNOS-positive neurons does not exceed that of motor neurons, which, at this stage of the disease, is ~50% in these transgenic mice (Gurney et al., 1994; Kostic et al., 1997*a*,*b*). Thus, it is most likely that the observed loss of nNOS immunoreactivity results from the actual loss of neurons in this animal model; the loss of nNOS immunoreactivity is consistent with neurons expressing nNOS dying as they also expressed mutant SOD1. It is interesting that it also appears that the rare remaining nNOS-positive neurons, as well as the neuropil, in the anterior horn of end-stage transgenic mSOD1 mice exhibited a more intense labeling as compared with their counterparts in age-matched wild-type animals (Fig. 2E and F). This suggests that the motor neurons that are spared may be those that exhibit the strongest nNOS immunostaining to start with (see above comment on variability of nNOS labeling in motor neurons) and/or have up-regulation of nNOS. Therefore, neurons with high levels of nNOS expression may be less vulnerable to the neurodegenerative process than those that have low levels of expression. This view has been put forward already to explain the relative vulnerability of different subsets of neurons in ALS (Wetts and Vaughn, 1998). Alternatively, we cannot exclude the possibility that dying motor neurons are in fact those with the highest nNOS expression. In support of this idea is the demonstration that injured motor neurons, indeed, induce nNOS (Wu et al., 1994; Estévez et al., 1998), which, in turn, may contribute to their own subsequent demise. nNOS enzymatic activity in the spinal cord of end-stage transgenic mSOD1 mice was also reduced to a magnitude that is consistent with that of motor neuronal loss (Fig. 5). It should be pointed out, however, that the spinal cord activity of nNOS in

end-stage transgenic mSOD1 mice, although markedly reduced, remains quite substantial and still about six times greater than the maximal activity of iNOS (Fig. 5). It is thus unlikely that the observed reduction in nNOS enzymatic activity would be sufficient to cause a shortage in NO formation and be deleterious. On the other hand, as the changes in the spinal cord of nNOS activity appeared to parallel that of motor neuron number, nNOS activity may be a useful marker of spinal cord neurodegeneration in this mouse model of ALS.

Another noteworthy aspect related to our data is the fact that at no disease stage did we observe nNOS immunoreactivity in glial cells in the spinal cord of transgenic mSOD1 mice. This is in striking contrast with the study of Cha et al. (1998), who reported in end-stage transgenic mSOD1 mice, and not in control mice, nNOS labeling in cells that were GFAP-positive and exhibited the typical morphology of astrocytes; of note, precedent exists for nNOS induction in neurons (Young and Ciampoli, 1998), but this would be, to our knowledge, the first example of nNOS induction in glial cells. Relevant to this apparent discrepancy is the fact that, in mice, at least three alternative spliced forms of nNOS exist: nNOSα, nNOSβ, and nNOSγ (Eliasson et al., 1997). Our anti-nNOS antibody, which is directed against the N-terminal 200 amino acids of nNOS, will only recognize the full-length and most abundant nNOSα form, but not the two other truncated and less abundant spliced variants, because they lack these amino acids (Eliasson et al., 1997). Conversely, the anti-nNOS antibody of Cha et al. (1998), which is directed against a region of nNOS (amino acid 1,414–1,429) that is conserved among the three forms (Eliasson et al., 1997), will recognize all three. Should this speculation be correct, it would indicate that, in the brain, whereas nNOSα is indeed constitutive and specific to neurons, nNOSβ and nNOSγ may be up-regulated and expressed in nonneuronal cells under pathological situations associated with gliosis.

In conclusion, the present study provides compelling evidence that the expression of iNOS, and possibly of nNOS, is altered in transgenic mSOD1 mice. It is thus plausible that, over the course of the disease, the observed NOS deregulation contributes to increasing the local concentration of NO, thereby promoting neurodegeneration. Recent studies indicate that the majority of NO-mediated neurotoxicity is dependent upon local "factors" produced within the susceptible target neurons (Dawson and Snyder, 1994). Relevant to this is the demonstration that, over the course of the disease, transgenic mSOD1 mice also develop mitochondrial electron transport chain defects (Browne et al., 1998), which, in turn, can stimulate the production of superoxide radicals (Halliwell and Gutteridge, 1991). NO will readily react with the superoxide radical to produce the potent oxidant, peroxynitrite (Beckman, 1994), at a rate three times faster than the reaction rate of SOD in catalyzing the dismutation of the superoxide radical to hydrogen peroxide (Beckman, 1994). Accordingly, a simple increase in the concentration of either NO, as speculated above, or superoxide radical, via the mentioned mitochondrial defect, can lead to the formation of peroxynitrite within the motor neurons (Beckman et al., 1990) and subsequent cell injury. Although peroxynitrite can cause direct injury to the cell (Dawson and Snyder, 1994), it also decomposes to the reactive hydroxyl radical (Beckman et al., 1994), which leads to further injury. In addition, peroxynitrite can nitrate tyrosine residues (Beckman et al., 1992), which cause major functional alterations to proteins (Ara et al., 1998). In support of this cascade of deleterious events being relevant to ALS pathogenesis are the demonstrations that the concentrations of hydroxyl radical and nitrotyrosine are, indeed, increased in the spinal cord of the transgenic mSOD1 mice (Beal et al., 1997; Ferrante et al., 1997; Bogdanov et al., 1998; S. Przedborski and M. Shigenaga, unpublished data). We therefore believe that our study, together with findings of other investigators (Beal et al., 1997; Ferrante et al., 1997), should revive the debate about the role of NO in the pathogenesis of ALS (Sasaki et al., 1996). We also believe that our data warrant additional studies to explore in-depth the effects of NOS inhibition, by mean of drugs or engineered animals, on motor neuron loss in transgenic mSOD1 mice, as they may open new therapeutic avenues aimed at stopping or slowing the progression of the disease in ALS.

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α-Synuclein Up-Regulation in Substantia Nigra Dopaminergic Neurons Following Administration of the Parkinsonian Toxin MPTP

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Abstract: Mutations in α-synuclein cause a form of familial Parkinson's disease (PD), and wild-type α-synuclein is a major component of the intraneuronal inclusions called Lewy bodies, a pathological hallmark of PD. These observations suggest a pathogenic role for α-synuclein in PD. Thus far, however, little is known about the importance of α-synuclein in the nigral dopaminergic pathway in either normal or pathological situations. Herein, we studied this question by assessing the expression of synuclein-1, the rodent homologue of human α-synuclein, in both normal and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-intoxicated mice. In normal mice, detectable levels of synuclein mRNA and protein were seen in all brain regions studied and especially in ventral midbrain. In the latter, there was a dense synuclein-positive nerve fiber network, which predominated over the substantia nigra, and only few scattered synuclein-positive neurons. After a regimen of MPTP that kills dopaminergic neurons by apoptosis, synuclein mRNA and protein levels were increased significantly in midbrain extracts; the time course of these changes paralleled that of MPTP-induced dopaminergic neurodegeneration. In these MPTP-injected mice, there was also a dramatic increase in the number of synuclein-immunoreactive neurons exclusively in the substantia nigra pars compacta; all synuclein-positive neurons were tyrosine hydroxylase-positive, but none coexpressed apoptotic features. These data indicate that synuclein is highly expressed in the nigrostriatal pathway of normal mice and that it is up-regulated following MPTP-induced injury. In light of the synuclein alterations, it can be suggested that, by targeting this protein, one may modulate MPTP neurotoxicity and, consequently, open new therapeutic avenues for PD. **Key Words:** Synuclein—MPTP—Neurodegeneration—Parkinson's disease—Substantia nigra—Dopaminergic neurons.
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Parkinson's disease (PD) is a common disabling neurodegenerative disorder that can present as both a familial and a nonfamilial (i.e., sporadic) condition (Fahn, 1988). Its cardinal clinical features include tremor, stiffness, and slowness of movement, all of which are attributed to the dramatic loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Fahn, 1988). Although the actual cause of PD remains unknown, a breakthrough on this question emerged from studies on the small brain-specific protein α-synuclein. The first clue linking α-synuclein to PD comes from the observation that point mutations in the α-synuclein gene cause an autosomal dominant parkinsonian syndrome almost indistinguishable from the prominent sporadic form of PD (Polymeropoulos et al., 1997; Kruger et al., 1998). The two missense mutations identified thus far result in a single amino acid substitution in α-synuclein protein, that is, an alanine being replaced by a hydrophobic residue threonine, at position 53, and proline, at position 30. Since the discovery of these mutations, data have been accumulated suggesting that both mutations may alter α-synuclein's normal intracellular distribution, enhance α-synuclein's propensity to interact with other intracellular proteins, and increase α-synuclein disposition to aggregate and consequently to form intraneuronal inclusions (Conway et al., 1998; El-Agnaf et al., 1998; Engelender et al., 1998; Giasson et al., 1999; Narhi et al., 1999). To date, efforts to identify α-synuclein mutations in sporadic PD have failed (Golbe, 1999). On the other hand, in sporadic PD, α-synuclein has been demonstrated to be a major component of the intraneuronal inclusions, Lewy bodies (LB), which are a pathological

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Abbreviations used: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LB, Lewy bodies; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NHS, normal horse serum; PBS, phosphate-buffered saline; PD, Parkinson's disease; SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulata; TBS, Tris-buffered saline; TH, tyrosine hydroxylase.

hallmark of the disease (Spillantini et al., 1997, 1998). Furthermore, oxidative stress, which is a leading pathogenic hypothesis of sporadic PD, has been reported to affect wild-type α-synuclein, causing the oxidatively damaged wild-type α-synuclein to mimic some of the abnormal behaviors of mutant α-synuclein (Hashimoto et al., 1999). These observations strongly suggest that both mutant and posttranslationally modified wild-type α-synuclein may participate in the SNpc dopaminergic neuron degeneration in PD, whether it is familial or sporadic.

α-Synuclein is a small ubiquitous protein highly expressed in presynaptic structures of, apparently, all neuronal pathways of the brain (Maroteaux and Scheller, 1991; Clayton and George, 1998; Lavedan, 1998). In light of its presumed role in synaptic function, α-synuclein has been studied especially in the brain of various animal species during development and more specifically during the time frame of synaptogenesis (Withers et al., 1997; Petersen et al., 1999). In humans, α-synuclein has also been studied intensively in brain regions such as cerebral cortex because of the possible role of one of its internal fragments called NAC (i.e., non-β-amyloid component) in the formation of plaques in Alzheimer's disease brains (Iwai et al., 1995, 1996; Irizarry et al., 1996; Masliah et al., 1996). However, although the association of mutant α-synuclein with at least some forms of PD is well established, our knowledge about the role of α-synuclein in normal or even injured SNpc dopaminergic neurons is, to date, quite poor. Therefore, to acquire better understanding about the relationship between α-synuclein and SNpc dopaminergic neurons, we studied the expression and distribution of synuclein-1, the rodent homologue of human α-synuclein (both referred to henceforth as α-synuclein, except when indicated), in normal mice. We also performed these investigations in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD to explore the response of α-synuclein to an injury and examine its contribution to the dopaminergic neurodegenerative process. We elected to use the MPTP model because, to date, it is recognized as the best experimental model of sporadic PD, replicating most of the biochemical and pathological features seen in the clinical condition (Przedborski and Jackson-Lewis, 1998).

MATERIALS AND METHODS

Animals

Eight-week-old male C57/bl mice (22–25 g; Charles River Breeding Laboratories, Wilmington, MA, U.S.A.) were used. Animals were housed three per cage in a temperature-controlled room under a 12-h light/12-h dark cycle with free access to food and water. Mice used in this study were treated according to the NIH *Guidelines for the Care and Use of Laboratory Animals* and with the approval of Columbia University's Institutional Animal Care and Use Committee.

MPTP administration

Mice were divided into two groups and received either a chronic or an acute MPTP regimen. For the *chronic regimen* (kills dopaminergic neurons by apoptosis; see Tatton and Kish, 1997), mice received one intraperitoneal injection of MPTP-HCl per day (30 mg/kg/day of free base; Research Biochemicals, Natick, MA, U.S.A.) for 5 consecutive days and were killed at 0, 1, 2, 4, 7, 14, 21, and 42 days after the last injection; control mice received saline injections only. Both saline- and MPTP-treated animals were then divided into two groups. The first group was perfused and the brains used for immunohistochemistry, whereas the second group of mice were killed and the brains quickly removed, dissected (midbrain, striatum, cerebellum, and cortex), snap-frozen on dry ice, and stored at −80°C for western blot and RT-PCR analysis. For the *acute regimen* (kills dopaminergic neurons by necrosis; see Jackson-Lewis et al., 1995), mice received on the day of the experiment four intraperitoneal injections of MPTP-HCl (20 mg/kg) in saline at 2-h intervals and were killed at 0, 2, 4, 7, and 21 days after injection; control mice received saline injections only. Both saline- and MPTP-treated animals were prepared for immunohistochemistry and western blot analysis as described above.

Western blot analysis

Total tissue proteins were isolated in 50 m*M* Tris-HCl, pH 7.0, 150 m*M* NaCl, 5 m*M* EDTA, 1% sodium dodecyl sulfate, 1% Nonidet P-40, and protease inhibitors (Mini Cocktail; Roche Diagnostics, Indianapolis, IN, U.S.A.). Protein concentration was determined using the bicinchoninic acid kit (Pierce, Rockford, IL, U.S.A.). After boiling in 1× Laemmli buffer, 50–100 μg of protein was loaded onto 12–15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose or polyvinylidene difluoride membrane, and blocked with 5% nonfat dry milk in 1× Tris-buffered saline (TBS), 0.1% Tween 20 for 1 h. Incubation with one of the primary antibodies was performed overnight at 4°C using 1:1,000 anti-synuclein-1 (Transduction Laboratories, Lexington, KY, U.S.A.), 1:1,000 β-synuclein (gift from Dr. S. Nakajo, Tokyo, Japan), 1:2,000 anti-synaptophysin (gift from Dr. Honer, Albert Einstein College of Medicine, Bronx, NY, U.S.A.), or 1:500 anti-tyrosine hydroxylase (anti-TH; Eugene Tech, Ridgefield Park, NJ, U.S.A.). Incubation with a secondary anti-mouse or anti-rabbit-conjugated horseradish peroxidase antibody was performed at room temperature for 1 h. After washing in 1× TBS, 0.1% Tween-20, blots were exposed to Super Signal Ultra chemiluminescence (Pierce) and exposed to Kodak β-Max film. Films were then digitized, each band was outlined with a screen cursor driven by a hand-held mouse, and optical densities were determined using a computerized image analysis system (Inquiry image analyzer, Loats Associates, Westminster, MD, U.S.A.).

Immunohistochemistry

After being anesthetized with pentobarbital (30 mg/kg i.p.), saline- (n = 5) and MPTP-treated mice (0, 1, 2, 4, 7, 14, 21, and 42 days after the last MPTP injection, n = 4–7 for each time point) were perfused intracardially with 24 ml of saline followed by 72 ml of cold 4% (wt/vol) paraformaldehyde in 0.1 *M* phosphate-buffered saline (PBS), pH 7.1. Animals were then decapitated, and brains were removed, immersed for 72 h in the same 4% paraformaldehyde fixative, and cryoprotected in 30% sucrose in 0.1 *M* PBS for 48 h at 4°C. Brains were then frozen on dry ice-cooled isopentane and stored at −80°C until use. Serial coronal sections (30 μm thickness) spanning the entire midbrain and the mid striatum were cut on a cryostat, collected free-floating in PBS, and processed as described below.

For all immunostaining, sections were first rinsed (3 × 5 min) with 0.1 *M* PBS, pH 7.4. Sections were then immersed in a solution of 3% H_2O_2/10% methanol for 5 min, followed by incubation with 5% normal horse serum (NHS) for 60 min. Sections were then incubated with the primary antibody (anti-synuclein-1, 1:1,000) in 0.1 *M* PBS, pH 7.4, containing 2% NHS and 0.3% Triton X-100, for 48 h at 4°C on a shaker. After rinsing in PBS, biotinylated secondary horse anti-mouse IgG (1:200; Vector, Burlington, CA, U.S.A.) in 0.1 *M* PBS, pH 7.4, containing 2% NHS was added, and the sections were incubated for 60 min at room temperature. This was followed by a final incubation in avidin/biotin peroxidase complex (Vector) for 60 min. Visualization was performed by incubation in 3,3′-diaminobenzidine/glucose/glucose oxidase for 10 min. All sections were then washed 3 × 5 min in PBS, mounted on 0.1% gelatin-coated slides, dried, dehydrated in graded ethanols, cleared in xylenes, and coverslipped. To test the specificity of the immunostaining, control sections were processed in an identical manner but with the primary or secondary antibody omitted. Adjacent sections were immunostained for TH (1:1,000; Calbiochem, San Diego, CA, U.S.A.) and counterstained with thionin.

To examine the colocalization of α-synuclein with TH, a double-immunofluorescence technique was used. After washing, sections were blocked in 5% normal goat serum and NHS in 0.1 *M* TBS for 1 h. Incubation with primary antibodies was performed for 48 h at 4°C, with anti-synuclein-1 (1:200) and anti-TH (1:500) antibodies. As the anti-TH antibody was made in rabbit and the anti-synuclein-1 antibody was made in mouse, we used different secondary antibodies for double labeling: anti-rabbit IgG labeled with Texas Red, and biotinylated anti-mouse IgG, followed by avidin D labeled with fluorescein. Sections were examined on green, red, and double (green + red) filters using confocal microscopy. To colocalize α-synuclein staining with apoptotic morphology, additional fluorescent α-synuclein-stained sections were counterstained with the DNA dyes TOTO-3 iodide (2 μ*M* in the secondary antibody solution) or Hoechst 33342 (10 μg/ml in phosphate buffer for 45 min) (both from Molecular Probes, Eugene, OR, U.S.A.).

Quantitative morphology

Early and late in the course of the MPTP neurodegenerative process, the number of α-synuclein-positive cells was minimal or none. This rendered unreliable the use of our stereological method (Mandir et al., 1999) because this approach requires a minimal number of cells being "countable" by applying the random paradigm. If the number is too small, we may miss the rare positive cells and count no cells that may be false. Thus, if this situation occurs, we will return to our assumption-based method (Przedborski et al., 1996), which is not affected by the rarity of the counted cells, following the recommendations of the editors of the *Journal of Comparative Neurology* (Coggeshall and Lekan, 1996) on the question. We are also aware that our conclusions regarding counted cells using this assumption-based method should only imply whether there are significantly more or less counted cells in the experimental cases. By applying these strict criteria, the above-cited editorial (Coggeshall and Lekan, 1996) indicates that our assumption-based method of cell counts is valid. In brief, counts were performed manually and blinded to the treatment received (i.e., MPTP or saline). For each mouse (n = 4–7 per group), eight different stereotaxic planes encompassing the entire substantia nigra and containing the SNpc were analyzed (interaural 0.88 to 0.16 mm; Franklin and Paxinos, 1997) by scanning the entire SNpc on both sides (light microscopy; ×200). The average number of neurons in each plane was added to provide a measure of the total number of SNpc α-synuclein-positive neurons for each animal and then divided by the number of counted sections to provide the number of α-synuclein-positive cells per section.

RNA extraction and RT-PCR

Total RNA was extracted from midbrain, striatal, and cerebellar samples from saline- and chronic MPTP-treated animals (at 0, 4, 7, 21, and 42 days after last MPTP injection, n = 5 for each time point) using a Qiagen RNA isolation kit (Qiagen, Valencia, CA, U.S.A.). The yield and quality of the RNA were determined by measuring the absorbance at 260 and 280 nm in a spectrophotometer. First-strand cDNA was synthesized from 1 μg of total RNA by reverse transcription, using the SuperScript Preamplification System with SuperScript II RNase H-reverse transcriptase (GibcoBRL Life Technologies, Grand Island, NY, U.S.A.). The reaction mixture (20 μl) for PCR consisted of 1 μl of cDNA template, 18 μl of Supermix (GibcoBRL), 0.01 pmol of [^{32}P]dCTP (New England Nuclear, Boston, MA, U.S.A.; specific activity, 3,000 Ci/mmol), and 4–10 pmol of each specific primer. The primer sequences were 5′-GTGGTTCATGGAGTGACAAC-3′ (forward) and 5′-AGGCTTCAGGCTCATAGTCT-3′ (reverse) for α-synuclein and 5′-AGAGAGGACCAGTCAGCCAA-3′ (forward) and 5′-TGACCAGAACCTTCTCTCAAGC-3′ (reverse) for synaptophysin. As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was coamplified using primer sequences 5′-GTTTCTTACTCCTTGGAGGCCAT-3′ (forward) and 5′-TGATGACATCAAGAAGTGGTGAA-3′ (reverse). Each PCR cycle consisted of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 1.5 min, followed by a final 10-min extension at 72°C. PCR amplification was carried out for 27 cycles for α-synuclein and synaptophysin and 22 cycles for GAPDH using a Perkin–Elmer GeneAmp 9700 thermal cycler. The conditions for each PCR amplification resulted in an exponential amplification range for quantification of each mRNA. After amplification, samples were separated on 5% polyacrylamide gel electrophoresis in 0.5× Tris–borate–EDTA buffer. Gels were dried and exposed overnight to a phosphorimager screen, and then radioactivity was quantified using a computerized analysis system (Bio-Rad Phosphorimager system).

Statistical analysis

For each experiment, four to seven mice per group were studied and all values are expressed as the means ± SEM. Differences were analyzed using one-way ANOVA with the different groups of mice as the independent factor. When ANOVA showed significant differences, pairwise comparisons were tested by Newman–Keuls post-hoc analysis. In all analyses, the null hypothesis was rejected at the 0.05 level.

RESULTS

Increased α-synuclein protein expression in the ventral midbrain after chronic MPTP intoxication

To determine whether α-synuclein may be involved in the deleterious cascade of events induced by MPTP, we assessed α-synuclein protein expression levels in the ventral midbrain of MPTP-intoxicated mice, at different time points after MPTP administration, using western blot analysis. The immunoblot for α-synuclein resulted in a single band of 19 kDa, as previously described

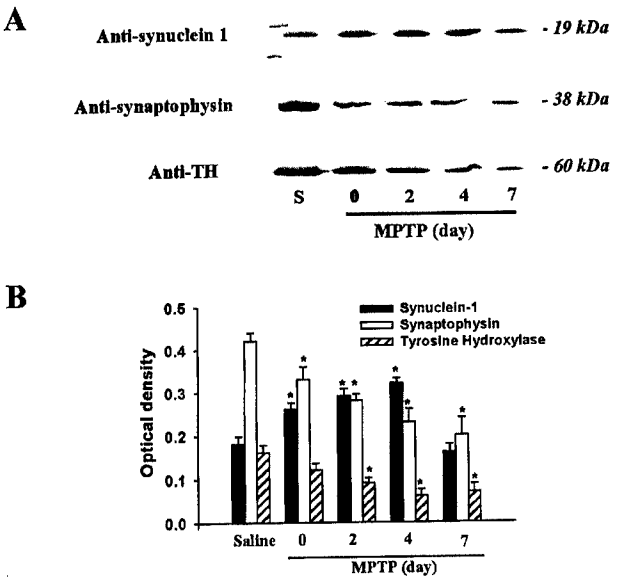

FIG. 1. Expression of synuclein-1, synaptophysin, and TH proteins in ventral midbrain samples of saline-treated (S) and chronic MPTP-intoxicated mice at different time points after intoxication. The immunoblot for synuclein-1 resulted in a single band of 19 kDa (**A**), confirming the specificity of the antibody. Quantitative results (**B**) were obtained by measurement of the optical density of each band using a computerized image analysis system as described in Materials and Methods. After chronic MPTP regimen, α-synuclein protein expression increased progressively in midbrain extracts from 0 to 4 days after MPTP administration, and then returned to the level of controls. Parallel to changes in α-synuclein protein expression, levels of synaptophysin progressively decreased in the ventral midbrain after chronic MPTP administration. Similarly, TH protein expression was also decreased after MPTP intoxication, reflecting the progressive loss of dopaminergic neurons. No changes in α-synuclein protein levels were detected in the striatum or in other cerebral structures, such as cerebellum or cortex (data not shown). $^*p < 0.05$, compared with saline (Newman–Keuls post-hoc analysis).

(Ueda et al., 1993). Quantitative results were obtained by measuring the optical density of each band using a computerized image analysis system as described in Materials and Methods. After the chronic MPTP regimen, α-synuclein protein expression progressively increased in midbrain extracts from 0 to 4 days after MPTP administration (Fig. 1). This increase was already significant at 0 days (+44%), peaked at 4 days (+77%), and then returned to control level. No changes in α-synuclein protein levels were detected in the striatum or in other cerebral structures, such as cerebellum or cortex (data not shown). In addition to α-synuclein, levels of synaptophysin, another presynaptic protein, were determined to test the possibility that α-synuclein alterations might be part of a nonspecific response of synaptic-related proteins to MPTP injury. In contrast to changes in α-synuclein protein levels, synaptophysin protein levels progressively decreased in the ventral midbrain after chronic MPTP administration (Fig. 1). No change of expression was detected in another member of the synuclein family, β-synuclein. TH protein levels were also decreased after chronic MPTP intoxication, reflecting the progressive loss of dopaminergic neurons. In contrast to the chronic regimen of MPTP, the acute regimen did not result in any significant change in α-synuclein protein expression.

Regional and cellular localization of α-synuclein up-regulation

To determine whether the observed changes in α-synuclein protein expression within the ventral midbrain were specific to the SNpc, we performed immunohistochemistry with the same anti-synuclein-1 antibody as used for the western blot. In saline-injected mice, there was a dense network of α-synuclein-positive nerve fibers over the entire substantia nigra that predominated in the pars reticulata (SNpr) and especially in its most medial and ventral parts. Superimposed on this dense α-synuclein-positive neuropil, there were also a few scattered α-synuclein-positive cells with a definite neuronal morphology in the SNpc (Fig. 2). Within α-synuclein-positive neurons, immunostaining was distributed diffusely over the somata with greater immunoreactivity in the cytoplasmic than in the nuclear area, and appeared to extend to proximal neuronal processes. After chronic, but not acute, MPTP intoxication, we did not observe any alteration at the level of α-synuclein-positive neuropil staining in the SNpc and SNpr. In contrast, after this MPTP regimen, the number of α-synuclein-positive neurons in the SNpc increased dramatically as early as 0 days after the last MPTP injection (i.e., 5 days after the first MPTP injection) (Fig. 2); the number of α-synuclein-positive neurons in the SNpc returned to baseline (i.e., saline controls) by 21 days after MPTP administration (data not shown). Of note, in neither saline- nor MPTP-injected mice did we observe (a) any immunostaining in the absence of the primary anti-synuclein-1 antibody, (b) any α-synuclein-positive intraneuronal aggregate, or (c) any glial cells exhibiting α-synuclein immunoreactivity.

Adjacent sections immunostained with TH and counterstained with thionin confirmed the cellular loss induced by MPTP intoxication, as previously described (Jackson-Lewis et al., 1995; Tatton and Kish, 1997). Furthermore, thionin staining revealed the presence of apoptotic morphology in the SNpc of chronic MPTP-intoxicated animals (Fig. 2). None of these apoptotic features could be colocalized with α-synuclein immunoreactivity with absolute certainty.

Double immunofluorescence was performed to identify the phenotype of neurons expressing α-synuclein. Examination with confocal microscopy revealed that all α-synuclein-positive neurons in the SNpc colocalized with TH immunoreactivity (Fig. 3), suggesting that α-synuclein up-regulation after MPTP occurs specifically within dopaminergic neurons, even if not all TH-positive neurons were immunostained with α-synuclein. Adjacent sections were used to colocalize fluorescent α-synuclein staining with apoptotic morphologies, using

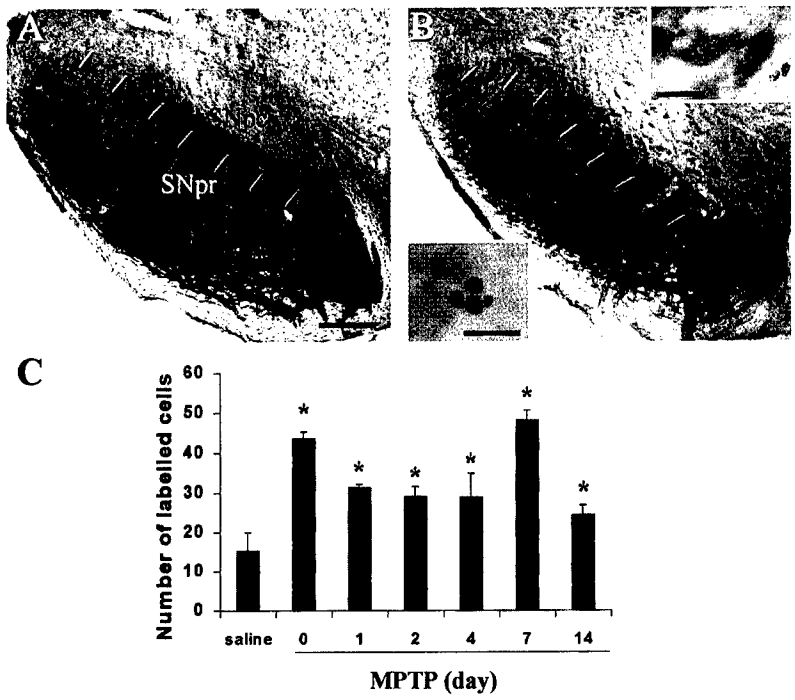

FIG. 2. Representative photomicrographs illustrating synuclein-1 immunoreactivity (brown) in the ventral midbrain of saline-treated (**A**) and chronic MPTP-intoxicated (**B**) mice, counterstained with thionin (blue). Quantitative results (**C**) were obtained by counting α-synuclein-labeled neurons in the SNpc. A strongly immunostained fiber network was observed in the SNpr in both saline-treated and MPTP-intoxicated animals (A and B). A small number of α-synuclein-immunoreactive neuronal bodies were detected in the SNpc of control saline-injected mice (A and C). After chronic, but not acute, MPTP intoxication, the number of α-synuclein-positive neurons in the SNpc increased dramatically (B and C), with peaks at 0 (as shown in B) and 7 days after intoxication. The number of α-synuclein-immunoreactive neurons progressively returned to levels similar to that of controls after 21 days (data not shown). α-Synuclein staining was regularly distributed within the neuronal cytosol, as seen at higher magnification in the upper inset in B, with no detectable intraneuronal inclusions. Up-regulation of α-synuclein expression was parallel to an apoptotic mode of neuronal death in the SNpc, as illustrated in the lower inset in B, with the presence of apoptotic morphology (with chromatin clumps) detected by thionin staining. No colocalization of apoptotic morphology with α-synuclein immunostaining was detected. *$p < 0.05$, compared with saline (Newman–Keuls post-hoc analysis). Scale bars = 200 μm (A), 40 μm (upper inset in B), and 10 μm (lower inset in B).

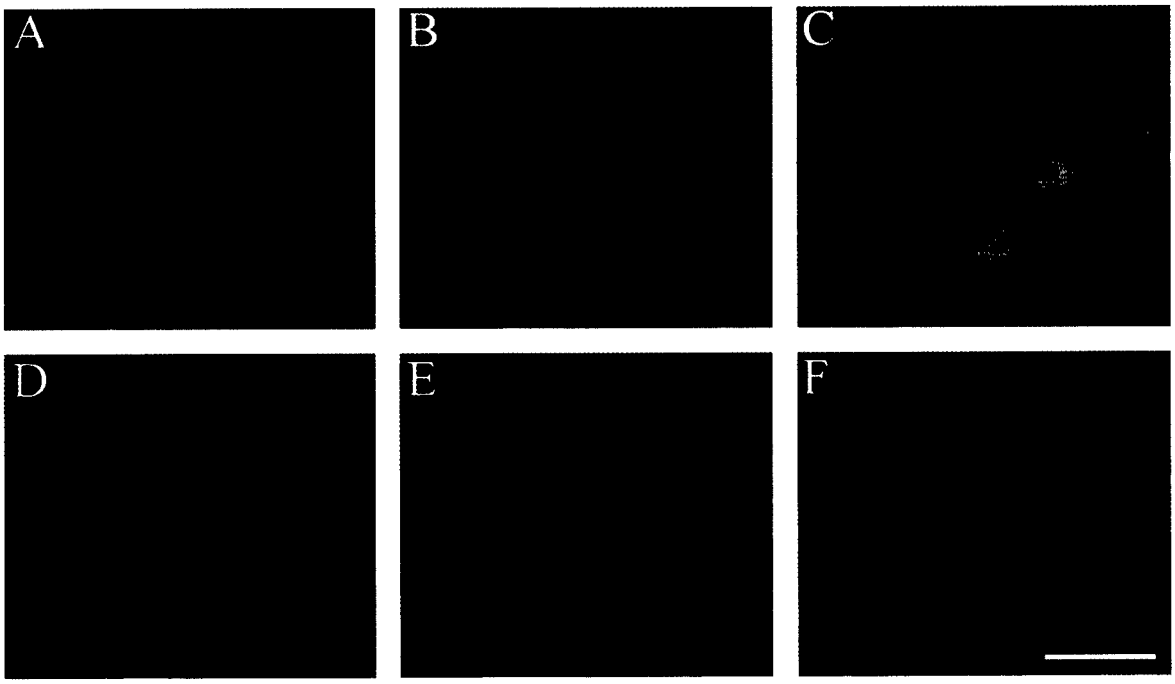

FIG. 3. Representative photomicrographs illustrating double-immunofluorescence staining, visualized with confocal microscopy, of synuclein-1 (green, B and E) and TH (red, A and D) in the SNpc of saline-injected mice (**D–F**) and after chronic MPTP administration (at 0 day after last MPTP injection) (**A–C**). In chronic MPTP-treated animals, α-synuclein immunoreactivity was colocalized with TH, as seen by double filter (red + green) in C. In saline-injected animals, very few α-synuclein-positive neurons were detected by immunofluorescence (E), as illustrated by a predominance of TH (red)-labeled cells using double filter (F). Scale bar = 50 μm.

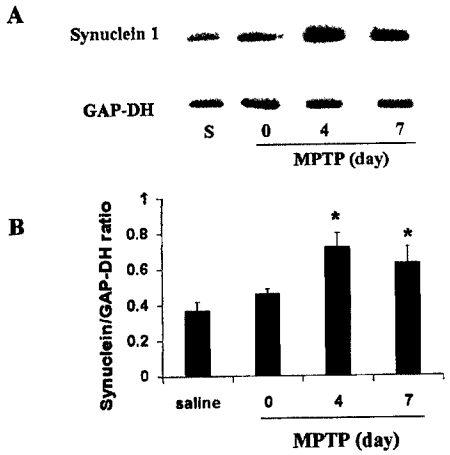

FIG. 4. Midbrain α-synuclein mRNA expression detected by RT-PCR. A photograph of the gel (**A**) and the corresponding bar graph (**B**) show increased α-synuclein mRNA expression after chronic MPTP administration compared with that in saline-injected mice. Levels of α-synuclein mRNA expression progressively decreased to the level of controls up to 21 and 42 days (data not shown). GAPDH mRNA expression levels were determined as an internal control and used to normalize the values for α-synuclein mRNA expression. No changes in α-synuclein mRNA expression were found in either the striatum or the cerebellum, and expression levels of synaptophysin mRNA were unchanged after MPTP intoxication (data not shown). $^*p < 0.05$, compared with saline (Newman–Keuls post-hoc analysis).

TOTO-3 iodide and Hoechst 33342 DNA dyes, failing to reveal any definite colocalization.

Up-regulation of α-synuclein mRNA following MPTP administration

To determine whether changes in α-synuclein protein expression are accompanied by changes in α-synuclein mRNA levels, additional midbrain samples of chronic MPTP-treated mice were used for semiquantitative RT-PCR amplification. Other cerebral structures, such as the striatum and the cerebellum, were also analyzed. In parallel, expression levels of synaptophysin mRNA were also determined. As an internal control, the mRNA levels of expression of the housekeeping gene GAPDH were determined and used to normalize the levels of α-synuclein and synaptophysin mRNA.

Consistent with α-synuclein protein alterations, α-synuclein mRNA was increased in the midbrain following chronic MPTP intoxication in a time-dependent manner (Fig. 4), peaking at 4 days (+94%) after intoxication and then progressively decreasing to the level of controls up to 21 days. No changes in α-synuclein mRNA expression were found in either the striatum or the cerebellum. Expression levels of synaptophysin mRNA in the ventral midbrain were either unchanged or decreased, although not statistically significant, after MPTP intoxication (data not shown).

DISCUSSION

In the present study, we first report the regional and cellular distribution of α-synuclein within the SNpc, which, to date, has not been studied. In normal mice, a striking aspect is the conspicuous α-synuclein-positive nerve fiber network that covers the entire substantia nigra, in the presence of only rare α-synuclein-positive neuronal cell bodies. This remarkable dissociation of α-synuclein immunoreactivity between nerve fibers and cell bodies is consistent with its known normal intracellular distribution in various regions of the mature brain in other animal species and is in keeping with its presumed synaptic function (Maroteaux and Scheller, 1991; Clayton and George, 1998; Lavedan, 1998). At the cellular level, most of the immunoreactivity was located in the cytoplasm of α-synuclein-positive perikarya and proximal processes, with some also present in the nuclei. Although we did not formally study other structures of the basal ganglia, robust α-synuclein immunostaining was also observed in the striatum, which showed high immunoreactivity, followed by the subthalamic nucleus, the globus pallidus, and the thalamus, all of which showed moderate-to-low immunoreactivity. This regional and cellular pattern of α-synuclein immunostaining agrees with that previously reported (Maroteaux and Scheller, 1991; Tobe et al., 1992; Irizarry et al., 1996; Lavedan, 1998), although the originally described nuclear localization of α-synuclein immunoreactivity, which we also observed in neurons of the normal mouse SNpc, has not been observed by all investigators.

This study also defines the topographical and temporal relationship between α-synuclein expression and neuronal degeneration following MPTP administration by using two different regimens of MPTP injections: one, called acute, kills SNpc dopaminergic neurons by necrosis, whereas the other, called chronic, kills SNpc dopaminergic neurons by apoptosis (Jackson-Lewis et al., 1995; Tatton and Kish, 1997). It appeared important to ascertain the α-synuclein response in the context of these two morphologically defined modes of cell death, as they represent the two current schools of thought regarding forms of cell death in PD (Burke and Kholodilov, 1998). Indeed, for many years, the consensus has been that, in PD, as in any pathological brain condition, neurons die by necrosis. However, biochemical and morphological features of apoptosis have been identified in parkinsonian, but not in control, postmortem brain samples (Burke and Kholodilov, 1998). This has raised the possibility that apoptosis may be a mode of neuronal death not only during development and morphogenesis, but also in diseased mature brain as in PD. Thus, it is interesting that α-synuclein expression appeared dramatically altered in the mouse brain following only the chronic, and not the acute, regimen of MPTP. The discrepancy in the α-synuclein response to the acute and chronic regimen cannot be explained by the dose of toxin used because we and others have demonstrated that one injection of 30 mg/kg/day for 5 consecutive days (i.e., chronic regimen) or four injections of 20 mg/kg every 2 h in 1 day (i.e., acute regimen) cause, in both cases, a loss of SNpc dopaminergic neurons that is, in this strain of mice, ~60% (Jackson-Lewis et al., 1995; Tatton and

Kish, 1997). The difference cannot be explained by the time points studied either, as those cover the SNpc dopaminergic neurodegeneration produced by both the chronic and acute regimen (Jackson-Lewis et al., 1995; Tatton and Kish, 1997). Therefore, at this point the most parsimonious explanation may be that α-synuclein alteration in the SNpc is associated specifically with the occurrence of apoptosis. Along this line, it is relevant to point out that the time course of α-synuclein up-regulation paralleled that of neuronal death produced by the chronic MPTP regimen, by either preceding or coinciding with the wave of actual cell degeneration. It is also important to mention that changes in α-synuclein expression followed the regional specificity of MPTP neurotoxic effects because α-synuclein mRNA and protein expression was increased in ventral midbrain, and specifically in dopaminergic neurons of the SNpc, but not in other structures, such as the cortex or cerebellum. The absence of detectable α-synuclein alterations in the striatum, despite definite MPTP-induced damage to SNpc projecting nerve fibers in this structure, likely reflects the small percentage that dopaminergic terminals represent in the striatum relative to the total pool of terminals (Descarries et al., 1996). On the other hand, increased α-synuclein expression does not seem to be part of a common response of synaptic-related proteins to MPTP injury because the expression levels of synaptophysin, another synapse-associated protein, were either unchanged or decreased after chronic MPTP intoxication. It is also noteworthy that the increase in the number of α-synuclein-immunoreactive cells lasted longer than the increases in the levels of α-synuclein mRNA and protein. Indeed, following MPTP injection, levels of α-synuclein protein detected by western blot were close to the levels found in saline-injected controls by day 7, whereas the number of α-synuclein-positive neurons was still increased by this time point and only appeared to return to baseline after day 14. This can be explained by the fact that although neurons may remain unquestionably immunoreactive beyond day 7, their actual α-synuclein protein content may have already decreased significantly. Changes in α-synuclein expression within the dense α-synuclein-positive fiber network in the substantia nigra may also contribute to this discrepancy. It is also important to mention that α-synuclein, which is primarily a synaptic-associated protein, is initially produced in the cell body and then rapidly transported to the nerve terminals (Withers et al., 1997). Accordingly, as MPTP damages terminals first and most severely (Herkenham et al., 1991; Jackson-Lewis et al., 1995), it is possible that the apparent increased content of ventral midbrain α-synuclein protein and number of SNpc α-synuclein-positive neurons result from an impaired anterograde transport of the protein and its subsequent accumulation at the site of synthesis. Although this possibility cannot be excluded with certainty, the observation that levels of α-synuclein mRNA are also increased rather supports the view that following MPTP administration α-synuclein is up-regulated and not solely accumulated in the cell bodies of SNpc dopaminergic neurons.

Altogether our data raise the possibility that α-synuclein up-regulation, which occurs in the specific context of MPTP-induced apoptotic death in SNpc dopaminergic neurons, contributes to the cascade of deleterious events that ultimately kill these cells. For instance, in light of the synaptic location of α-synuclein, it may be envisioned that its up-regulation may affect the normal synaptic machinery, which, in turn, may disturb the trophic support of SNpc dopaminergic neurons originating from the striatum. This hypothesis is of particular relevance to apoptosis because, in many settings, deprivation of the target-derived trophic support triggers a massive apoptotic death among the projecting neurons (Burke and Kholodilov, 1998). By using a two-hybrid system, it has been demonstrated that α-synuclein can bind to intracellular proteins (Engelender et al., 1999). Although, among those, none seemed to belong to the large family of apoptotic-related proteins, it remains plausible that up-regulation of α-synuclein may alter the normal intracellular trafficking of certain proteins that, like many of the Bcl-2 family members, depend on being at a specific intracellular location to exert their regulatory effects on apoptosis (Merry and Korsmeyer, 1997). There is also compelling evidence to indicate that α-synuclein has a significant propensity to aggregate, and that this property can be enhanced by the familial PD-linked mutations or by posttranslational modifications, such as produced by oxidative stress (Conway et al., 1998; El-Agnaf et al., 1998; Giasson et al., 1999; Hashimoto et al., 1999; Narhi et al., 1999). Relevant to this is the fact that α-synuclein is present in high amounts in the intraneuronal inclusion LB, which is regarded by some as a key factor in the demise of SNpc dopaminergic neurons in PD (Spillantini et al., 1997, 1998). In the MPTP mouse model, we failed to identify any evidence supporting the formation of intraneuronal inclusions in SNpc dopaminergic neurons, whether they were α-synuclein-immunoreactive or not. This rules out the possibility that α-synuclein up-regulation, should it play a role in the MPTP-induced neurotoxic process, does so through the formation of LB-like inclusions. However, except for old-aged monkeys (Forno et al., 1988), it is known that MPTP does not stimulate the formation of LB-like inclusions, and thus the MPTP model may not be suitable to study the role of α-synuclein aggregation in the SNpc dopaminergic neurodegenerative process. Arguing against a role of α-synuclein up-regulation in the MPTP neurotoxic process is the fact that although we found a close regional and temporal relationship between α-synuclein up-regulation and induced neuronal death, we did not find any definite association at a cellular level between α-synuclein protein expression and morphological features of apoptosis: chromatin clumps, as evidenced by thionin or fluorescent DNA dyes (Clarke and Oppenheim, 1995; Suzuki et al., 1997), could not be unequivocally colocalized with α-synuclein immunoreactivity in

neurons. We cannot exclude, however, that at this advanced stage of cellular injury, there is not a loss of immunoreactivity, which may account for the difficulty in colocalizing apoptotic features with α-synuclein immunostaining. For example, it is notorious that only a small fraction of the SNpc apoptotic neurons seen during normal development retain their TH immunoreactivity (Oo and Burke, 1997). It should also be mentioned that apoptotic profiles found in a brain section at any given time following the chronic MPTP regimen are few in number and that apoptotic cells undergo a rapid turnover estimated at only a few hours (Oppenheim, 1991), making the likelihood of identifying both apoptotic features and α-synuclein immunoreactivity within the same neuron quite low.

As mentioned above, a main target of MPTP toxicity is the dopaminergic nerve terminals of the striatum (Herkenham et al., 1991; Jackson-Lewis et al., 1995). Therefore, one may speculate that our results, rather than being the expression of a death process, can represent the expression of a synaptic plasticity response. Supporting this view is the observation that the avian α-synuclein homologue synelfin is specifically up-regulated during early stages of song learning in zebra finch, suggesting a role for the synuclein family in shaping up the synaptic network (George et al., 1995). Furthermore, α-synuclein up-regulation in the substantia nigra during postnatal development in rats coincides with the time of maximal synaptogenic activity (Burke and Kholodilov, 1998); similar observations have been reported at the level of the hippocampus and cerebral cortex (Petersen et al., 1999). Conversely, we may argue against this plasticity hypothesis by emphasizing the fact that, in this case, we would have expected to see similar α-synuclein changes after the acute and chronic MPTP regimens, and not only in the levels of α-synuclein, but also in the levels of other synaptic proteins, such as synaptophysin.

Most of the α-synuclein-positive neurons had a healthy morphological appearance following MPTP administration. This observation may suggest a third possibility for α-synuclein up-regulation, that is, it represents a cellular attempt to survive MPTP injury. Consistent with this is the observation that α-synuclein up-regulation also seemed to involve surviving SNpc neurons in the model of programmed cell death induced by developmental striatal target lesion in rats (Burke and Kholodilov, 1998). In the context of Alzheimer's disease, it has been reported that surviving synapses in Alzheimer's brains contain a higher concentration of α-synuclein compared with controls (Masliah et al., 1996). Also relevant to the speculated beneficial role of α-synuclein up-regulation are the observations that α-synuclein is a potent inhibitor of phospholipase D2 (Jenco et al., 1998) and that, by nullification of phospholipase A2, which is a closely related member of this family, mice become resistant to MPTP (Klivenyi et al., 1998).

In summary, this study reports on alterations of α-synuclein expression in the MPTP mouse model of PD and its relationship to the mode of neuronal death, as well as the time course and the regional specificity of these changes. These data provide valuable descriptive information regarding α-synuclein in both the normal and the injured dopaminergic nigrostriatal pathway. In addition, the observed changes in α-synuclein expression, whether they are part of a death or, on the contrary, of a surviving response, bring to light significant alterations within the SNpc neurons that could be targeted for the development of new neuroprotective therapies for PD.

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ADVANCES IN OUR KNOWLEDGE OF MPTP ACTION AND MECHANISM

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SUMMARY

MPTP causes damage to substantia nigra pars compacta (SNpc) dopaminergic (DA) neurons as seen in Parkinson's disease (PD). After systemic administration of MPTP, its active metabolite, MPP^+, accumulates within SNpc DA neurons, where it inhibits ATP production and stimulates superoxide radical formation. The produced superoxide radicals react with nitric oxide (NO) to form peroxynitrite, a highly reactive tissue-damaging species that damages proteins by oxidation and nitration. Only selected proteins appear nitrated and among these, is found tyrosine hydroxylase (TH), the rate limiting enzyme in DA synthesis. The process of nitration inactivates TH and consequently DA production. Peroxynitrite also nicks DNA, which, in turn, activates poly(ADP-ribose) polymerase (PARP). PARP activation consumes ATP, and thus acutely depletes the cell energy stores. This latter event aggravates the pre-existing energy failure due to MPP^+-induced mitochondrial respiration blockade and precipitates cell death. Altogether, these findings support the view that MPTP's deleterious cascade of events include mitochondrial respiration deficit, oxidative stress, and energy failure. Because of the similarity between the MPTP mouse model and PD, it is tempting to propose that a similar scenario applies to the pathogenesis of PD.

INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disorder of unknown cause whose cardinal clinical features include shaking, stiffness, and slowness of movement. Most, if not all, of these disabling clinical abnormalities are attributed to a profound decrease in dopamine in the striatum which results from the dramatic loss

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of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Fahn, 1988). The prevalence of PD has been estimated at $\approx$ 1,000,000 in North America with $\approx$ 50,000 newly affected individuals each year. Thus far, the most potent treatment for PD remains the administration of a precursor of dopamine, L-DOPA, which, by replenishing the brain with dopamine, alleviates PD symptoms. However, the chronic administration of L-DOPA often causes motor and psychiatric side effects, which may be as debilitating as PD itself (Kostic et al., 1991). Furthermore, there is no supportive evidence that L-DOPA therapy impedes the progressive death of SNpc dopaminergic neurons. Therefore, without undermining the importance of L-DOPA therapy in PD, it remains essential to elucidate the cascade of events that underlie PD's neurodegenerative process. To this end and in light of the rarity of available post-mortem brain samples from PD patients, many investigators, including ourselves, have focused their research efforts on experimental models of PD such as the one produced by the parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).

MPTP — A MODEL OF PARKINSON'S DISEASE

The fact that MPTP causes a parkinsonian syndrome was discovered in 1982 when a group of drug addicts in California were rushed to the emergency room with a severe bradykinetic and rigid syndrome (Langston et al., 1983). Subsequently, thanks to some fine detective work, it was discovered that this syndrome was induced by the self-administration of street batches of a synthetic heroin analogue whose synthesis had been heavily contaminated by a by-product, MPTP (Langston and Irwin, 1986). In the period of a few days following the administration of MPTP, these patients exhibited a severe and irreversible akinetic rigid syndrome. The analogy to PD was rapidly made by Dr. Langston and his group, and levodopa was tried with great success, relieving the symptoms of these unfortunate patients.

Since the discovery that MPTP causes parkinsonism in human and non-human primates as well as in various other mammalian species, this neurotoxin has been used extensively as a model of PD (Heikkila et al., 1989; Kopin and Markey, 1988; Langston and Irwin, 1986). In human and non-human primates, MPTP produces an irreversible and severe parkinsonian syndrome that replicates almost all of the features of PD including tremor, rigidity, slowness of movement, postural instability, and even gait freezing. The responses as well as the complications to traditional anti-parkinsonian therapies are virtually identical to those seen in PD. However, while in PD it is believed that the neurodegenerative process occurs over several years, MPTP produces a clinical condition consistent with "end-stage PD" in a few days (Langston, 1987). Except for a single case (Davis et al., 1979), no human pathological material has been available. Thus, the comparison between PD and the MPTP model is largely limited to primates (Forno et al., 1993). From neuropathological data, MPTP administration causes damage to the dopaminergic pathways identical to that seen in PD (Agid et al., 1987) with a resemblance that goes beyond the degeneration of SNpc dopaminergic neurons. Like PD, MPTP causes a greater loss of dopaminergic neurons in the SNpc than in the ventral tegmental area (Muthane et al., 1994; Seniuk et al., 1990) and a greater degeneration of dopaminergic nerve terminals in the putamen than in the caudate nucleus (Moratalla et al., 1992). On the other hand, two typical neuropathologic features of PD have, until now, been lacking in the MPTP model. First, except for the SNpc, the other pigmented nuclei such as the locus coeruleus have been spared according to most published reports. Second,

the eosinophilic intraneuronal inclusions, called Lewy bodies, so characteristic of PD, have thus far not been convincingly observed in MPTP-induced parkinsonism (Forno et al., 1993). Also worth noting is the fact that post-mortem brain samples from PD patients (DiMauro, 1993) show a selective defect in the same mitochondrial electron transport chain complex that is affected by MPTP (Gluck et al., 1994; Nicklas et al., 1987). Abnormalities in parameters of oxidative stress in post-mortem PD brain tissue suggest that this disease is caused by an overproduction of free radicals (Przedborski and Jackson-Lewis, 1998), the same highly reactive tissue damaging species that are suspected of being involved in MPTP-induced dopaminergic toxicity *in vivo* (Hantraye et al., 1996; Przedborski et al., 1992; Schulz et al., 1995). However, despite this impressive resemblance between PD and the MPTP model, MPTP has never been recovered from post-mortem brain samples or body fluids of PD patients. Altogether, these findings are consistent with MPTP not causing PD, but being an excellent experimental model of PD. Accordingly, it can be speculated that elucidating of molecular mechanisms, MPTP, should lead to important insights into the pathogenesis and treatment of PD.

MODE OF ACTION OF MPTP

The metabolism of MPTP is a complex, multistep process (Przedborski and Jackson-Lewis, 1998). After its systemic administration, MPTP, which is highly lypophilic, rapidly crosses the blood-brain barrier. Once in the brain, the pro-toxin MPTP is metabolized to 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) by the enzyme monoamine oxidase B (MAO-B) within non-dopaminergic cells, and then (probably by spontaneous oxidation) to 1-methyl-4-phenylpyridinium (MPP^+), the active toxic compound. Thereafter, MPP^+ is released (by an unknown mechanism) in the extracellular space. Brain inflow of MPTP together with its transformation into MPP^+ determine the amount of MPP^+ available to enter dopaminergic neurons. The next important step in the MPTP neurotoxic pathway is the mandatory entry of MPP^+ into dopaminergic neurons. Since MPP^+ is a polar molecule, unlike its precursor MPTP, it cannot freely enter cells, but depends on the plasma membrane carriers to gain access to DA neurons. MPP^+ has a high affinity for plasma membrane dopamine transporter (DAT) (Mayer et al., 1986), as well as for norepinephrine and serotonin transporters. The obligatory character of this step in the MPTP neurotoxic process is demonstrated by the fact that blockade of DAT by specific antagonists such as mazindol (Javitch et al., 1985) or ablation of DAT gene in mutant mice (Bezard et al., 1999) completely prevents MPTP-induced toxicity. Conversely, transgenic mice with increased brain DAT expression are more sensitive to MPTP (Donovan et al., 1999).

Once inside dopaminergic neurons, MPP^+ can follow at least three routes: (i) it can bind to the vesicular monoamine transporters (VMAT) which will translocate MPP^+ into synaptosomal vesicles (Liu et al., 1992), (ii) it can be concentrated by an active process within the mitochondria (Ramsay and Singer, 1986), and (iii) it can remain in the cytosol and interact with different enzymes (Klaidman et al., 1993). The fraction of MPP^+ destined to each of these routes, is probably a function of MPP^+ intracellular concentration and affinity for VMAT, mitochondria carriers, and cytosolic enzymes. The importance of the vesicular sequestration of MPP^+ is demonstrated by the fact that cells transfected to express greater density of VMAT are converted from MPP^+-sensitive to MPP^+-resistant cells (Liu et al., 1992). Conversely, we demonstrated that mutant mice with 50% lower VMAT expression are significantly more

sensitive to MPTP-induced dopaminergic neurotoxicity compared to their wild-type littermates (Takahashi et al., 1997). Altogether, these findings indicate that there is a clear inverse relationship between the capacity of MPP^+ sequestration (i.e., VMAT density) and the magnitude of MPTP neurotoxicity.

Inside dopaminergic neurons, MPP^+ can also be concentrated by an active process within the mitochondria (Ramsay and Singer, 1986), where it impairs mitochondrial respiration by inhibiting complex I of the electron transport chain (Mizuno et al., 1987; Nicklas et al., 1985) through its binding at or near the same site as the mitochondrial poison rotenone (Higgins, Jr. and Greenamyre, 1996; Ramsay et al., 1991). The inhibition of complex I impedes the flow of electrons along the mitochondrial electron transport chain, leading to a deficit in ATP formation. It appears, however, that complex I activity should be reduced $> 70\%$ to cause severe ATP depletion (Davey and Clark, 1996) and that, in contrast to *in vitro*, *in vivo* MPTP causes only a transient 20% reduction in mouse striatal and midbrain ATP levels (Chan et al., 1991). These findings raise the question as to whether MPP^+-related ATP deficit can be the sole factor underlying MPTP-induced dopaminergic neuronal death. Another consequence of complex I inhibition by MPP^+ is an increased production of free radicals, especially of superoxide (Cleeter et al., 1992; Hasegawa et al., 1990; Rossetti et al., 1988). From the above-mentioned findings, it may be speculated that the initiation of MPP^+'s deleterious cascade of events may result from energy failure and oxidative stress, which individually may not be sufficient to kill cells, but in combination may well be lethal. A similar scenario of interplay among mitochondrial dysfunction, energy failure, and oxidative stress has been postulated for PD (Beal, 1995).

The importance of MPP^+-related superoxide production in dopaminergic toxicity process *in vivo* is demonstrated by the fact that transgenic mice with increased brain activity of copper/zinc superoxide dismutase (SOD1) are significantly more resistant to MPTP-induced dopaminergic toxicity than their non-transgenic littermates (Przedborski et al., 1992). This finding strongly suggests that superoxide radical plays a pivotal role in the MPTP neurotoxic process. However, superoxide is poorly reactive, and it is the general consensus that this radical does not cause serious direct injury (Halliwell and Gutteridge, 1991). Instead, superoxide is believed to exert many or most of its toxic effects through the generation of other reactive species such as hydroxyl radical, whose oxidative properties can ultimately kill cells (Halliwell and Gutteridge, 1991). For instance, superoxide facilitates hydroxyl radical production by hydrogen peroxide and transitional metals such as iron (i.e., Fenton reaction) (Halliwell and Gutteridge, 1991). Although this reaction can readily take place *in vitro*, its occurrence *in vivo* is subordinate to such factors as low pH (Liochev and Fridovich, 1994). Despite this unfavorable pH constraint, MPTP does stimulate the formation of hydroxyl radicals *in vivo*, as evidenced by the increase in the hydroxyl radical-dependent conversion of salicylate into 2,3- and 2,5-dihydroxy-benzoates (Schulz et al., 1995).

Superoxide can also react with NO to form peroxynitrite, another potent oxidant (Beckman et al., 1990). At physiological pH and in aqueous milieu, this reaction proceeds five times faster than the decomposition of superoxide by SOD (Huie and Padmaja, 1993). The intracellular concentration of SOD1 is estimated at $10 - 40\,\mu M$ (Wink et al., 1996). Thus, NO concentration has to be $\approx 10\,\mu M$ for peroxynitrite formation to be competitive, which is not unrealistic as NO production at the cellular level is estimated at $1 - 10\,\mu M$ (Beckman et al., 1990). The situation is different, however, for superoxide, whose basal intracellular concentration is low (Beckman et

al., 1994). Thus, under normal conditions, superoxide is limiting, and it is likely that minimal peroxynitrite formation occurs. Conversely, in pathological conditions, should superoxide concentrations increase, as in response to MPTP administration, formation of appreciable amounts of peroxynitrite is expected. In light of this and of our previous work on superoxide (Przedborski et al., 1992), we (Przedborski et al., 1996) and others (Hantraye et al., 1996; Schulz et al., 1995) have assessed the role of NO in the MPTP neurotoxic process. These studies show that inhibition of NOS attenuates, in a dose-dependent fashion, MPTP-induced striatal dopaminergic loss in mice (Przedborski et al., 1996; Schulz et al., 1995). We also demonstrate that 7-nitroindazole (7-NI), a compound that inhibits NOS activity without significant cardiovascular effects in mice (Moore et al., 1993), is profoundly neuroprotective against MPTP-induced SNpc dopaminergic neuronal death (Przedborski et al., 1996). The protective effect of the NOS antagonist 7-NI against MPTP-induced striatal and SNpc dopaminergic damage was subsequently demonstrated in monkeys (Hantraye et al., 1996).

MPTP PROPOSED MECHANISM OF ACTION

From the above findings, the following scheme can be proposed to explain both selectivity and dopaminergic toxicity: MPTP is converted to MPP^+ which is transported into dopaminergic neurons via the dopamine transporter. MPP^+ inhibits enzymes in the mitochondrial electron transport chain, resulting in ATP deficit and increased "leakage" of superoxide from the respiratory chain. Superoxide cannot readily transverse cellular membranes and so remains in the cell and organelle in which it is produced. In contrast, NO is membrane-permeable and diffuses into neighboring neurons. If the neighboring cell has elevated levels of superoxide, then there is an increased probability of superoxide reacting with NO to form peroxynitrite, which is highly reactive, damaging lipids, proteins, and DNA. In this scheme, it is the site of generation of superoxide which determines whether a cell will succumb to NO- and peroxynitrite-mediated deleterious effects. Since dopaminergic neurons selectively accumulate MPP^+, which in turn stimulates superoxide production, these neurons are selectively at risk.

SOURCE OF NO AND NO SYNTHASE

As summarized above, there is strong evidence that NO participates in the MPTP neurotoxic process. Because MPTP selectively kills dopaminergic neurons, it is expected that the deleterious cascade of events that underlie the neurodegeneration takes place inside dopaminergic neurons. There are experimental arguments to indicate that superoxide concentration is, indeed, increased inside dopaminergic neurons by MPP^+. However, NO is produced by NOS which, thus far, has not been identified inside dopaminergic neurons in rodents; although this needs to be confirmed, low levels of NOS might be present in dopaminergic neurons in humans (Bredt et al., 1991). In contrast to their lack of NOS, at least in rodents, dopaminergic structures are surrounded by NOS-containing fibers and cell bodies in the striatum, and, to a lesser extent, in the SNpc (Bredt et al., 1991; Leonard et al., 1995). Because NO is uncharged and lypophilic (Lancaster, 1996), it is able to travel away from its site of synthesis and inflict remote cellular damage without the need for any export mechanisms. It is suggested that NO, which is highly diffusible, can travel in random

directions up to $150 - 300\,\mu m$ during the $5 - 15$ sec that correspond to its estimated half-life in physiological and in aqueous conditions (Lancaster, 1996). Although this modeling may depart from the actual *in vivo* situation encountered by a molecule of NO, it gives credence to the hypothesis that NO can cover a distance several times greater than the diameter of a cell. We are thus speculating that the NO production involved in MPTP toxicity takes place in non-dopaminergic cells present in the vicinity of dopaminergic structures.

Another question pertinent to the origin of NO in the MPTP model is which isoforms of NOS are primarily involved in this process? Nitric oxide is formed from arginine by NOS which oxidizes the guanidino nitrogen of arginine, releasing NO and citrulline. To date, three distinct NOS isoenzymes have been purified and molecularly cloned: neuronal NOS (nNOS, NOS I), inducible NOS (iNOS, NOS II), and endothelial NOS (eNOS, NOS III). Since all three isoforms of NOS have been identified in the brain, each of these can individually or in combination be involved in the production of NO used in MPTP neurotoxic process.

NO SYNTHASE ISOFORMS

Neuronal NOS is the predominant isoform of NOS in the brain. Its catalytic activity and protein is identifiable throughout the brain (Bredt et al., 1991; Huang et al., 1993). Relevant to MPTP, nNOS is present in high density in the striatum within intrinsic medium-sized neurons co-localizing somatostatin and neuropeptide Y (Dawson and Dawson, 1996). In the midbrain, nNOS is found in cholinergic neurons and within serotoninergic fibers (Dawson and Dawson, 1996; Leonard et al., 1995). Thus, both by its abundance and its localization, nNOS appears to be an excellent candidate for producing NO for MPTP. In agreement with this is our demonstration that mutant mice deficient in nNOS are partially protected against MPTP-induced striatal dopaminergic toxicity (Przedborski et al., 1996). The finding that mice are better protected by the NOS antagonist 7-NI than by lacking nNOS expression suggests that nNOS is important, but may not be the sole isoform of NOS involved in this process. Can it be iNOS?

In the normal brain, iNOS is not detectable (Lowenstein et al., 1992) or is minimally expressed (Keilhoff et al., 1996). However, under pathological conditions, iNOS expression can significantly increase in activated astrocytes as well as in other cells such as microglia (Simmons and Murphy, 1992), invading macrophages. This was shown in the brain after kainic acid lesion (Wallace and Fredens, 1992), ischemic damage (Nakashima et al., 1995), or stab wound (Simmons and Murphy, 1992). A similar scenario may exist in the MPTP model. Indeed, from our recent data it appears that early in the course of MPTP-induced dopaminergic neuron degeneration there is an increase in midbrain iNOS activity within the strong astrocytic and microglial reaction that occurs in the SNpc following MPTP administration. This recent study also shows that changes in iNOS activity are already substantial 24 hr after MPTP administration, which precedes the peak of dopaminergic neurodegeneration (Jackson-Lewis et al., 1995). Therefore, NO derived from iNOS is likely minimal in normal brains, but may become increasingly substantial as MPTP-induced dopaminergic neurodegeneration progresses. Accordingly, iNOS may not play a significant role in the initiation of the MPTP toxic process, but may amplify it and assure its propagation by fueling dopaminergic neurons with increasing amounts of NO.

SUPEROXIDE AND NITRIC OXIDE

Superoxide is produced by many biological reactions, and especially by mitochondrial respiration (Halliwell and Gutteridge, 1991). It can be engaged in numerous reactions including direct oxidation of biological molecules (e.g., cathechols) and production of hydroxyl radicals. Similarly, NO exerts many biological effects that can be defined as direct (i.e., resulting from the reactions between NO and specific biological molecules) and indirect (i.e., resulting from the reactions between reactive nitric oxide species [RNOS], which are derived from NO oxidation, and specific biological targets) (Wink et al., 1996). Most, if not all, of NO's direct effects appear to be related to biological regulatory effects, and not to neurotoxicity (Wink et al., 1996); although NO can directly affect mitochondrial respiration *in vitro* (Bolaños et al., 1997), the deleterious consequence of this effect remains to be determined *in vivo*. Conversely, NO's indirect actions, which are mediated by RNOS such as nitrite (NO_2^-), nitrate (NO_3^-), and peroxynitrite and its protonated derivative, peroxynitrous acid (N_2O_3H), are unquestionably deleterious (Wink et al., 1996); in aqueous conditions, RNOS such as NO^+ and NO^- rapidly react with water and, thus, are unlikely to be major participants in noxious reactions.

PEROXYNITRITE AND NITROTYROSINE

In light of the above, it appears that, since they are weak oxidants, neither superoxide nor NO is, by itself, sufficiently damaging to participate directly in the MPTP toxic process. In contrast, we have also presented above different arguments supporting peroxynitrite in this role. The versatility of peroxynitrite as an oxidant is impressive (Beckman, 1994; Uppu et al., 1996). For instance, an important aspect of peroxynitrite's deleterious action is the oxidation of phenolic rings in proteins, and in particular of tyrosine residues (Ohshima et al., 1990), to form nitrotyrosine as the most important product (Van der Vliet et al., 1996). As such, detection and quantification of nitrotyrosine is important indirect evidence that peroxynitrite is involved in a pathological process. Relevant to the participation of peroxynitrite in the MPTP model, it has been demonstrated that MPTP significantly increases striatal levels of **free nitrotyrosine** in mice (Schulz et al., 1995). Although this finding provides major impetus to the implication of peroxynitrite in the MPTP model, one should be aware that the relationship between free and protein nitrotyrosine is unknown, and the physiopathologic role, if any, of free nitrotyrosine remains to be determined.

Aside from being a marker, nitrotyrosine can be a harmful modification as it can inactivate enzymes and receptors that depend on tyrosine residues for their activity (Ischiropoulos et al., 1992; Trotti et al., 1996) and prevent phosphorylation of tyrosine residues important for signal transduction (Kong et al., 1996; Martin et al., 1990). This described cascade of events appears quite relevant to MPTP's mode of action as we have demonstrated that, following MPTP administration to mice, both striatal and midbrain levels of nitrotyrosine in proteins increase in a time-dependent fashion and that tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, becomes inactivated by tyrosine nitration (Ara et al., 1998).

DNA DAMAGE AND POLY(ADP-RIBOSE) POLYMERASE

Thus far, the lion share of attention has been on the effects of reactive species produced after MPTP administration on proteins. However, as stated above, most of the reactive species that may be implicated in the MPTP model, like peroxynitrite, can damage, through oxidative processes, many vital cellular elements other than proteins (Halliwell and Gutteridge, 1991). Among these, DNA is of unique importance, because it is the repository of genetic information and is present in single copies. Oxidants like peroxynitrite can cause a range of DNA damage (Halliwell and Gutteridge, 1991). For example, DNA exposed to peroxynitrite produces 8-hydroxyguanine and 8-hydroxydeoxyguanosine; two modifications whose levels seem increased in midbrain of post-mortem PD brains compared to normal controls (Alam et al., 1997; Sanchez-Ramos et al., 1994). Because peroxynitrite can nitrate the aromatic group, it can also stimulate the formation of 8-nitrodeoxyguanosine (Byun et al., 1999). Finally, intact cells exposed to peroxynitrite exhibit a dose-dependent increase in DNA single strand breakage (Szabo et al., 1996), which is another important type of DNA alteration. In light of the proposed oxidant species involved in MPTP neurotoxicity, all of these DNA modifications can possibly occur in this model, as well as in PD. However, despite the potential pathological role of DNA damage, to date we are not aware of any published study on this process in the MPTP model. Nevertheless, we have preliminary data generated in collaboration with Dr. M.F. Chesselet (Department of Neurology, UCLA) indicating that MPTP does indeed cause conspicuous DNA damage such as strand breaks in SNpc neurons of MPTP-treated mice.

Although all of the aforementioned modifications are potentially mutagenic and thus likely harmful, strand break is especially attractive because of its link with the enzyme poly(ADP-ribose) polymerase (PARP). Indeed, DNA single strand breakage is an obligatory trigger for the activation of PARP, a phenomenon that we believe, for the reasons that follow, to be a major factor in the overall MPTP-induced cascade of deleterious events. Thus far, the actual functions of PARP remain uncertain, and data obtained with cell-free systems and cells from PARP knockout mice suggest that, contrary to the common belief, PARP would not have direct role in DNA repair mechanisms (Lindahl et al., 1995; Szabo, 1996).

On the other hand, it is clear that the activation of PARP results in the cleavage of NAD^+ into ADP-ribose and nicotinamide, both *in vitro* and *in vivo* (Lindahl et al., 1995; Szabo, 1996). In turn, PARP covalently attaches ADP-ribose to diverse proteins, including nuclear proteins, histones, and PARP itself. PARP then extends the initial ADP-ribose groups into a nucleic acid group-like polymer, poly(ADP-ribose). It is, therefore, manifest that PARP activation, by synthesizing poly(ADP-ribose) polymer, can rapidly deplete intracellular stores of NAD^+ which may impair glycolysis and mitochondrial electron transport chain activities, and, consequently, ATP formation (Lindahl et al., 1995; Szabo, 1996). This PARP-dependent cascade of events could play a critical role in the demise of the SNpc dopaminergic neurons as suggested by *in vitro* and *in vivo* data (Byun et al., 1999; Cosi and Marien, 1998; Zhang et al., 1995). This scenario may be even more significant if, as in the case of the MPTP model, the production of ATP in SNpc dopaminergic neurons is already compromised due to the inhibition of the mitochondrial complex I by MPP^+ (Chan et al., 1991). In favor of the importance of PARP activation in the MPTP neurotoxic process *in vivo* is our demonstration that mutant mice deficient in PARP are more resistant to MPTP-induced dopaminergic neuronal death (Mandir et al., 1999).

CONCLUSION

The current understanding of the MPTP mode of action proposes that MPP^+ causes oxidative stress mediated by superoxide and NO. This leads to damage of proteins, lipids, and DNA, all contributing to major cellular dysfunctions. In addition, activation of reparative DNA enzymes, which consume ATP, aggravate the already reduced pool of ATP caused by the action of MPP^+ on complex I. This will impair numerous vital cellular reactions that are ATP-dependent. Subsequently, oxidative stress- and energy failure-related damage affect the cell's ability to maintain intracellular potential. Accordingly, intracellular potential will progressively rise until it reaches the threshold of activation of glutamate N-methyl-D-aspartate ionophor-channel and consequently triggers an excitotoxic insult (Turski et al., 1991). This suggests that MPTP-induced cell death results from a complex interplay among mitochondrial dysfunction, oxidative stress, energy failure, and excitotoxicity. All of these key players will contribute to the impaired function of the cell until it comes incompatible with life, and thus the cell dies by necrosis (Jackson-Lewis et al., 1995), if the injury is so severe that no cellular function is preserved, or by apoptosis (Tatton and Kish, 1997), if the injury is less severe and some cellular function is preserved. Because of the close similarity between PD and the MPTP model, it is likely that a similar cascade of deleterious events underlies the pathogenesis of PD.

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The role of glial cells in Parkinson's disease

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Parkinson's disease is a common neurodegenerative disorder characterized by the progressive loss of the dopaminergic neurons in the substantia nigra pars compacta. The loss of these neurons is associated with a glial response composed mainly of activated microglial cells and, to a lesser extent, of reactive astrocytes. This glial response may be the source of trophic factors and can protect against reactive oxygen species and glutamate. Aside from these beneficial effects, the glial response can mediate a variety of deleterious events related to the production of reactive species, and pro-inflammatory prostaglandin and cytokines. This article reviews the potential protective and deleterious effects of glial cells in the substantia nigra pars compacta of Parkinson's disease. Curr Opin Neurol 14:483–489.

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Abbreviations

GDNF	glial-derived neurotrophic factor
GFAP	glial fibrillary acid protein
IL-1β	interleukin-1β
iNOS	inducible nitric oxide synthase
MPP^+	1-methyl-4-phenylperydinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NOS	nitric oxide synthase
SNpc	substantia nigra pars compacta
TNF-α	tumor necrosis factor-α

1350-7540

Introduction

Parkinson's disease is a common neurodegenerative disorder characterized mainly by resting tremor, slowness of movement, rigidity, and postural instability [1•] and associated with a dramatic loss of dopamine-containing neurons in the substantia nigra pars compacta (SNpc) [2]. The number of Parkinson's disease patients has been estimated at ~1 000 000 in North America, with ~50 000 newly affected individuals each year [1•]. Thus far, the most effective treatment for Parkinson's disease remains the administration of a precursor of dopamine, L-dopa, which, by replenishing the brain in dopamine alleviates almost all Parkinson's disease symptoms. However, the chronic administration of L-dopa often causes motor and psychiatric side effects, which may be as debilitating as Parkinson's disease itself [3], and there is no supportive evidence that L-dopa therapy impedes the neurodegenerative process in Parkinson's disease. Therefore, without undermining the importance of L-dopa therapy in Parkinson's disease, there is an urgent need to acquire a deeper understanding of both etiologic (i.e., causes) and pathogenic (i.e., mechanisms of cell death) factors implicated in Parkinson's disease, not only to prevent the disease, but also to develop therapeutic strategies aimed at halting its progression. To elucidate such factors, and consequently to develop new therapies, the neuropathology of Parkinson's disease has been revisited in search of abnormalities that could shed light on these putative culprits. In keeping with this goal, it is worth mentioning that aside from the dramatic loss of dopamine neurons, the SNpc is also the site of a glial reaction in both Parkinson's disease and experimental models of Parkinson's disease [4–7]. Gliosis is a recognized prominent neuropathological feature of many diseases of the brain, whose sole and unique function has been thought, for many years, to be the removal of cellular debris. Since then, mounting evidence indicates that the role played by gliosis in pathological situations may not be restricted to its 'housekeeping' function but may also include actions which significantly and actively contribute to the demise of neurons, especially in neurodegenerative diseases like Parkinson's disease. Interestingly, several lines of evidence demonstrate that gliosis may behave in a 'yin and yang' fashion because, depending upon the situation, it may mediate either beneficial or harmful events. In this review, we will summarize the observations regarding gliosis in Parkinson's disease and in experimental models of Parkinson's disease as well as outline recent findings regarding the potential role of

gliosis in the overall neurodegenerative process that occurs in Parkinson's disease.

As a preamble to our review, it is important to remind the reader that glia is composed of macroglia, including astrocytes and oligodendrocytes, and microglia. As mentioned by Wilkin and Knott [8], so far, oligodendrocytes, which are involved in the process of myelination, have not been implicated in Parkinson's disease, whereas both astrocytes and microglial cells have. Accordingly, the focus of this section will be on astrocytes and microglial cells. Astrocytes are crucial, in the normal, undamaged adult brain, to the homeostatic control of the neuronal extracellular environment [8]. Conversely, little is known about microglial functions in the normal brain. Following an injury to the brain, both astrocytes and microglial cells undergo various phenotypic changes that enable them to respond to and to play a role in the pathological processes [9,10]. For instance, microglial activation is characterized by proliferation, increased or de-novo expression of marker molecules, such as major histocompatibility complex antigens, migration, and eventually changes into a macrophage-like appearance [11].

Glial reaction in Parkinson's disease

In normal brains, neither resting astrocytes nor microglial cells are evenly distributed [12,13]. For instance, density of microglial cells is remarkably higher in the substantia nigra compared to other midbrain areas and brain regions such as hippocampus [14]. This observation, together with the finding that substantia nigra neurons are much more susceptible to activated microglial-mediated injury [14], lend support to the idea that gliosis may play an especially meaningful role in Parkinson's disease.

The nigrostriatal pathway is the most affected dopaminergic system in Parkinson's disease. The neurons that form this pathway have their cell bodies in the SNpc and their nerve terminals in the striatum. Of particular relevance to this review is the finding that the loss of dopaminergic neurons in post-mortem parkinsonian brains is associated with a significant glial reaction [4,5,15,16]. Interestingly, however, while the damage to dopaminergic elements is consistently more severe in the striatum than in the SNpc, the response of glial cells is consistently more robust in the SNpc than in the striatum [5]. This discrepancy can be explained by the fact that dopaminergic structures are in dominance in the SNpc whereas they are in a minority in the striatum (e.g., dopamine synapses represent $<15\%$ of the entire pool of synapses in the striatum). Aside from this topographical difference, the magnitude of the astrocytic and microglial responses in parkinsonian brains are also very different. The SNpc of many but not all post-mortem Parkinson's disease patients exhibit, at best, a mild increase in the number of astrocytes and in the immunoreactivity for glial fibrillary acid protein (GFAP) [4,16]. Despite these changes, full-blown reactive astrocytes have been observed only in a few instances [4,16]. Of note, the density of GFAP-positive astrocytes appears to be inversely related to the magnitude of dopaminergic neuronal loss across the different main dopaminergic areas of the brain in Parkinson's disease post-mortem samples [12], suggesting that dopaminergic neurons within areas poorly populated with astrocytes are more prone to degenerate. Conversely, among the astrocytic pathologic features seen in Parkinson's disease, what does correlate positively with the severity of SNpc dopaminergic neuronal loss is the count of α-synuclein positive-inclusions within SNpc astrocytes [17]; whether these inclusions have any pathogenic significance remains unknown. Unlike the astrocytic response, the activation of microglial cells in Parkinson's disease is consistently dramatic [5,15,16]. Microscopically, this microglial response in the SNpc culminates in those sub-regions most affected by the neurodegenerative process [5,15,16]. Moreover, activated microglial cells are predominantly found in close proximity to free neuromelanin in the neuropil and to remaining neurons, onto which they sometimes agglomerate to produce an image of neuronophagia [5]. In 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-intoxicated individuals, post-mortem examination reveals a marked glial reaction in the SNpc whose magnitude seems to parallel that of dopaminergic neuronal loss [18••]. In all three autopsy cases, both reactive astrocytes and activated microglial cells as well as images of neuronophagia are abundantly seen in the SNpc [18••].

The aforementioned studies indicate that the glial response in the SNpc is fairly similar between humans with Parkinson's disease and those intoxicated by MPTP, although a more significant astrocytic reaction is seen in the latter [18••]. From a neuropathological standpoint, microglial activation and especially neuronophagia is indicative of an active, ongoing process of cell death. While this contention is consistent with the fact that Parkinson's disease is a progressive condition, it challenges the notion that MPTP produces a 'hit-and-run' kind of damage and rather suggests that a single acute insult in the SNpc could set in motion a self-sustaining cascade of events with long-lasting deleterious effects. It remains that neither human post-mortem Parkinson's disease studies nor MPTP cases provide information about the temporal relationship between the loss of dopaminergic neurons and the glial reaction in the SNpc. Looking at mice injected with MPTP and killed at different time points thereafter, it appears that the time course of reactive astrocyte formation parallels that of dopaminergic structure destruction in both the striatum and the SNpc, and that GFAP expression

remains upregulated even after the main wave of neuronal death has passed [6,19,20••]. These findings suggest that, in the MPTP mouse model [21•], the astrocytic reaction is secondary to the death of neurons and not the reverse. This is supported by the demonstration that blockade of 1-methyl-4-phenylperydinium (MPP^+, the active metabolite of MPTP [21•]) uptake into dopaminergic neurons not only completely prevents SNpc dopaminergic neuronal death but also GFAP upregulation [22]. Remarkably, activation of microglial cells, which is also quite strong in the MPTP mouse model [6,19,20••,23], occurs much earlier than that of astrocytes and, more importantly, reaches a maximum before the peak of dopaminergic neurodegeneration [20••]. In light of the MPTP data presented above, it can be surmised that the response of both astrocytes and microglial cells in the SNpc clearly occurs within a timeframe allowing these glial cells to participate in the demise of dopaminergic neurons in the MPTP mouse model and possibly in Parkinson's disease. In the following sections, we will examine through which beneficial or detrimental mechanisms the glial response in Parkinson's disease can possibly play out in the neurodegenerative process.

The protective effect of glial cells in Parkinson's disease

As mentioned above, glial response to injury may in fact have beneficial effects which, in the case of Parkinson's disease, could attenuate neurodegeneration. Among the different mechanisms by which glial-derived neuroprotection could be mediated, the first that comes to mind involves the production of trophic factors.

To date, it is well recognized that many mature and, even more so, immature tissues and cell types, including glial cells, possess trophic properties that are essential for the survival of dopaminergic neurons. Relevant to this is the observation that striatal oligodendrocyte-type 2 astrocytes greatly improve the survival and phenotype expression of mesencephalic dopaminergic neurons in culture, while simultaneously decreasing the apoptotic demise of these neurons [24]. Although the actual identity of this glial-related trophic factor remains to be established, several others have already been well characterized. Among those, glial-derived neurotrophic factor (GDNF), which can be released by activated microglia, seems to be the most potent factor in supporting SNpc dopaminergic neurons during their period of natural developmental death in postnatal ventral midbrain cultures [25]. It is also worth emphasizing that GDNF induces dopaminergic nerve fiber sprouting in the injured rodent striatum [26], and that this effect is markedly decreased when GDNF expression is inhibited by intrastriatal infusion of antisense oligonucleotides [27]. Furthermore, GDNF, delivered either by infusion of the recombinant protein or by viral vectors, has been shown to markedly attenuate dopaminergic neuronal death and to significantly boost dopaminergic function within injured neurons in both MPTP-treated monkeys and mice [28,29••,30]. Unfortunately, in humans with Parkinson's disease, much less enthusiastic results have been obtained thus far, in that repetitive intraventricular injections of recombinant GDNF to one advanced parkinsonian patient was poorly tolerated and failed to halt the progression of the disease [31].

Glial cells may also protect dopaminergic neurons against degeneration by scavenging toxic compounds released by the dying neurons. For instance, dopamine can produce reactive oxygen species through different routes [32•]. Along this line, glial cells may protect remaining neurons against the resulting oxidative stress by metabolizing dopamine via monoamine oxidase-B and catechol-*O*-methyltransferase present in astrocytes, and by detoxifying reactive oxygen species through the enzyme glutathione peroxidase, which is detected almost exclusively in glial cells [33•]. Glia, which can avidly take up extracellular glutamate, may mitigate the presumed harmful effects of the subthalamic excitotoxic input to the substantia nigra [34]. Taken together, the data reviewed here support the contention that glial cells could have neuroprotective roles in Parkinson's disease. Whether any of those, however, actually dampen the neurodegenerative process in parkinsonian patients remains to be demonstrated.

The deleterious role of glial cells in Parkinson's disease

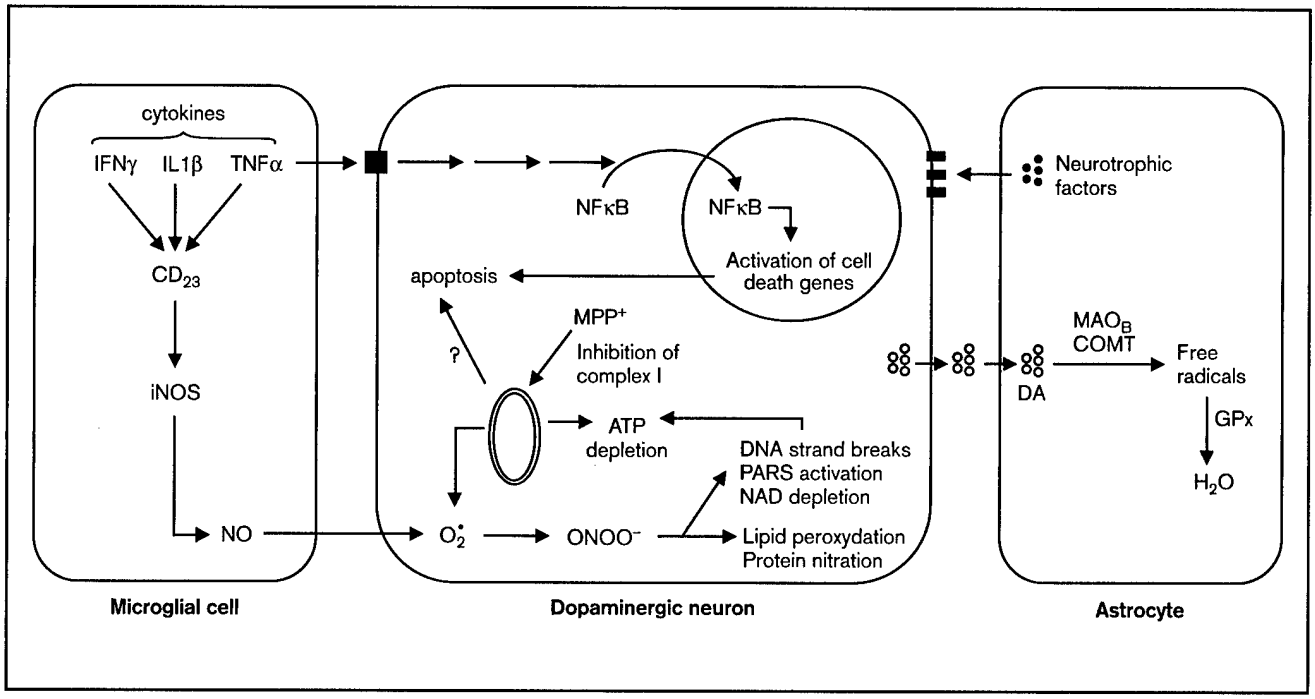
As we will see now, there are also many compelling findings which support the contention that glial cells could be harmful in Parkinson's disease. In this context, the spotlight appears to be more on activated microglial cells and less on reactive astrocytes. The importance of activated microglial cells in the neurodegenerative process is underscored by the following demonstrations in rats [35]: (1) the stereotaxic injection of bacterial endotoxin lipopolysaccharide into the SNpc causes a strong activation of microglia throughout the substantia nigra, followed by a marked degeneration of dopaminergic neurons; and (2) the pharmacological inhibition of microglial activation prevents lipopolysaccharide-induced SNpc neuronal death.

Activated microglial cells can produce a variety of noxious compounds including reactive oxygen species, reactive nitrogen species, pro-inflammatory prostaglandins, and cytokines. Among the array of reactive species, lately, the lion's share of attention has been given to reactive nitrogen species due to the prevailing idea that nitric oxide-mediated nitrating stress could be pivotal in

the pathogenesis of Parkinson's disease [36,37,38•–40•]. So far, however, none of the characterized isoforms of nitric oxide synthase (NOS) has been identified in SNpc dopaminergic neurons; hence, nitric oxide involved in the nitrating stress of Parkinson's disease most likely originates from other neurons and/or glial cells, as we hypothesized previously [36]. It is thus particularly relevant to mention that numerous glial cells in the SNpc of both Parkinson's disease patients [41•] and MPTP-treated mice [20••,23], but not of controls, express high levels of inducible NOS (iNOS). This NOS isoform, upon its induction, produces high amounts of nitric oxide for a prolonged period of time [42], as well as superoxide radicals [43], two reactive species which can either directly or indirectly promote neuronal death.

Prostaglandins and their synthesizing enzymes, such as cyclooxygenase type 2, constitute a second group of potential culprits. Indeed, cyclooxygenase type 2 has emerged as an important determinant of cytotoxicity associated with inflammation [44,45•]. In the normal brain, cyclooxygenase type 2 is significantly expressed only in specific subsets of forebrain neurons that are primarily glutamatergic in nature [46], which suggests a role for cyclooxygenase type 2 in the postsynaptic signaling of excitatory neurons. However, under pathological conditions, especially those associated with a glial response, cyclooxygenase type 2 expression in the brain can increase significantly, as does the level of its products (e.g., prostaglandin E_2), which are responsible for many of the cytotoxic effects of inflammation. Interestingly, cyclooxygenase type 2 promoter shares many features with iNOS promoter [42] and, thus, these two enzymes are often co-expressed in disease states associated with gliosis. Therefore, it is not surprising to find cyclooxygenase type 2 and iNOS expressed in SNpc glial cells of post-mortem Parkinson's disease samples [47]; prostaglandin E_2 content is also elevated in SNpc from Parkinson's disease patients [48]. Of relevance to the potential role of prostaglandin in the pathogenesis of Parkinson's disease is the demonstration that the pharmacological inhibition of both cyclooxygenase types 1 and 2 attenuates MPTP toxicity in mice [49].

Figure 1. Potential involvement of glial cells in the pathogenesis of Parkinson's disease



Activated microglial cells may contribute to dopaminergic neurodegeneration by releasing cytotoxic compounds such as cytokines. Cytokines may exert a direct effect on dopaminergic neurons by activating transduction pathways that lead to apoptosis or, alternatively, by inducing the expression of iNOS within glial cells and the subsequent formation of nitric oxide (NO). NO is membrane permeable and can diffuse to neighboring dopaminergic neurons. If the neighboring cell has elevated levels of superoxide ($O_2^{\bullet}$), there is an increased probability that superoxide will react with NO to form peroxynitrite ($ONOO^-$), which can damage lipids, proteins and DNA. Damaged DNA stimulates Poly(ADP-ribose) synthase (PARS) activity, which further contributes to the ATP depletion induced by the MPP^+-mediated inhibition of the mitochondrial complex I. Other glial cells, such as astrocytes, may have a neuroprotective effect on dopaminergic neurons by producing neurotrophic factors, such as GDNF, or by metabolizing dopamine (DA) by monoamino oxidase-B (MAO_B) or catechol-*O*-methyltransferase (COMT), then eliminating free radicals using glutathione peroxidase (GPx).

A third group of glial-derived compounds that can inflict damage in Parkinson's disease is the pro-inflammatory cytokines. Several among these, including tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), are increased in both SNpc tissues and cerebrospinal fluids of Parkinson's disease patients [50–52] although some of the reported alterations may be related to the chronic use of the anti-Parkinson's disease therapy L-dopa [53]. It remains that, at autopsy, convincing immunostaining for TNF-α, IL-1β, and interferon-γ is observed in SNpc glial cells from Parkinson's disease patients [54••]. These cytokines may act, at least, at two levels in Parkinson's disease. First, while they are produced by glial cells, they can stimulate other glial cells not yet activated, thereby amplifying and propagating the glial response and consequently the glial-related injury to neurons. Relevant to this scenario are the following demonstrations [54••]: glial-derived TNF-α, IL-1β, and interferon-γ activate other microglial cells which start to express the macrophage cell surface antigen FcεR11; now, activation of FcεR11 on these newly activated microglial cells induces iNOS expression and the subsequent production of nitric oxide which, in turn, can amplify the production of cytokines within glial cells (e.g., TNF-α) and can diffuse to neighboring neurons. Second, glial-derived cytokines may also act directly on dopaminergic neurons by binding specific cell surface cytokine receptors (e.g., TNF-α receptor). Once activated, these cytokine receptors trigger intracellular death-related signaling pathways whose molecular correlates include translocation of the transcription nuclear factor-κ-B from the cytoplasm to the nucleus and activation of the apoptotic machinery. In connection with this, Parkinson's disease patients exhibit a 70-fold increase in the proportion of dopaminergic neurons with nuclear factor-κ-B immunoreactivity in their nuclei compared to control subjects [55]. In relation to apoptosis, Bax, a potent pro-apoptotic protein, is upregulated after MPTP administration and its ablation prevents the loss of SNpc dopaminergic neurons in this experimental model [56]; and caspase-3, a key effector of apoptosis, is activated in post-mortem Parkinson's disease samples [57].

Conclusion

We have tried to succinctly review the issue of glial response in Parkinson's disease and how this cellular component of Parkinson's disease neuropathology, which has been neglected far too long, can play out in the overall neurodegenerative process (Fig. 1). Accordingly, key findings and, as often as possible, recent studies were included in our discussion to provide an up-to-date look at this question. Although we have tried to provide the reader with a balanced view of this issue, it is our opinion that, given the available evidence to date, data supporting a detrimental role of the glial response in Parkinson's disease outweigh those supporting a beneficial role. We also believe that, should the glial response in Parkinson's disease indeed be implicated in the neurodegenerative process, it is unlikely that any aspect of the glial response initiates the death of SNpc dopaminergic neurons, but quite possibly propagates the neurodegenerative process. This view, if confirmed, may thus have far-reaching therapeutic implications as targeting a specific aspect of the glial-related cascade of deleterious events may prove successful in slowing or even halting further neurodegeneration in Parkinson's disease [58].

Acknowledgements

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MPTP: a review of its mechanisms of neurotoxicity

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Abstract

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes damage to substantia nigra pars compacta (SNpc) dopaminergic (DA) neurons as seen in Parkinson's disease (PD). After systemic administration of MPTP, its active metabolite, MPP^+, accumulates within SNpc DA neurons, where it inhibits ATP production and stimulates superoxide radical formation. The produced superoxide radicals react with nitric oxide (NO) to produce peroxynitrite, a highly reactive tissue-damaging species that damages proteins by oxidation and nitration. Only selected proteins appear nitrated, and among these is found tyrosine hydroxylase (TH), the rate limiting enzyme in DA synthesis, and the pre-synaptic protein α-synuclein. Peroxynitrite also nicks DNA, which, in turn, activates poly(ADP-ribose) polymerase (PARP). PARP activation consumes ATP, and thus acutely depletes the cell energy stores. This latter event aggravates the preexisting energy failure due to MPP^+-induced mitochondrial respiration blockade and precipitates cell death. On the other hand, MPP^+ also activates highly regulated cell death-associated molecular pathways that participate in the relentless demise of neurons in PD. Altogether, these findings support the view that MPTP's deleterious cascade of events include mitochondrial respiration deficit, oxidative stress, energy failure and activation of apoptotic genetic programs. Because of the similarity between the MPTP mouse model and PD, it is tempting to propose that a similar scenario applies to the pathogenesis of PD.

Keywords: Free radicals; 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine; Neurodegeneration; Nitric oxide; Parkinson's disease; Poly(ADP-ribose) polymerase; Apoptosis; α-Synuclein

1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder of unknown cause whose cardinal clinical features include tremor, stiffness, slowness of movement, and postural instability [1]. Most, if not all, of these disabling clinical abnormalities are attributed to a profound decrease in brain dopamine content in the striatum which results from the dramatic loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) [1]. The prevalence of PD has been estimated at ~1 000 000 in North America with ~50 000 newly affected individuals each year. Thus far, the most potent treatment for PD remains the administration of a precursor of dopamine, L-DOPA, which, by replenishing the brain with dopamine, alleviates PD symptoms. However, the chronic administration of L-DOPA often causes motor and psychiatric side effects, which may be as debilitating as PD itself [2]. Furthermore, there is no supportive evidence that L-DOPA therapy impedes the progressive death of SNpc dopaminergic neurons. Therefore, without undermining the importance of L-DOPA therapy in PD, it is clear that symptomatic treatments are not the ultimate solution for PD patients. On the other hand, it is as clear that the ultimate solution will emanate from therapeutic strategies effective at stopping or slowing the neurodegenerative process of PD. To this end, it is essential to elucidate the cascade of deleterious events that underlie the demise of SNpc dopaminergic neurons in PD. Over the past 50 years or so, intense research efforts geared toward unraveling the etiopathogenesis of PD have employed experimental models of PD in an attempt to shed light onto this extremely serious problem. As a result, a plethora of both in vitro and in vivo models have emerged; without exception, by virtue of being experimental models, all have been praised for their resemblance to PD but, at the same time, have been harshly criticized for their shortcomings. An insightful discussion on this 'dual personality' of experimental models of PD can be found in Ref. [3]. The topic of this review will be devoted to the parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) which, in our opinion, remains to date the best

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experimental model of PD, especially in the context of studies designed to explore molecular mechanisms involved in the degeneration of SNpc dopaminergic neurons. Its superiority over other experimental models also stems, of course, from the fact that MPTP produces a PD-like syndrome in humans. Indeed, this fact unquestionably represents, in the eyes of investigators, physicians, and even patients, a huge impetus toward regarding the MPTP model as a credible tool in the study of PD, a situation not many other experimental models of PD can boast. In keeping with this, it cannot be stressed enough that, while the effect on humans is a 'plus' for the MPTP-model, it also raises the issue of safety, as accidental intoxication of laboratory personnel may have disastrous repercussions, a risk that can only be minimized if proper measures and precautions are implemented while using MPTP. Please refer to Ref. [4] for a comprehensive review on the topic of use and safety of MPTP.

2. MPTP as a model of PD

The fact that MPTP causes a parkinsonian syndrome was discovered in 1982 when a group of drug addicts in California were rushed to the emergency room with a severe bradykinetic and rigid syndrome [5]. Subsequently, it was discovered that this syndrome was induced by the self-administration of street batches of a synthetic meperidine analogue whose synthesis had been heavily contaminated by a by-product, MPTP [6]. In the period of a few days following the administration of MPTP, these patients exhibited a severe and irreversible akinetic rigid syndrome. The analogy to PD was rapidly made by Dr Langston and his group, and L-DOPA was tried with great success, relieving the symptoms of these patients.

Since the discovery that MPTP causes parkinsonism in human and non-human primates as well as in various other mammalian species, this neurotoxin has been used extensively as a model of PD [6–8]. In human and non-human primates, MPTP produces an irreversible and severe parkinsonian syndrome that replicates almost all of the features of PD including tremor, rigidity, slowness of movement, postural instability, and even gait freezing. The responses as well as the complications to traditional anti-parkinsonian therapies are virtually identical to those seen in PD. However, while in PD it is believed that the neurodegenerative process evolves over several years, the most-active phase of neuronal death is presumably completed in a short period of time following MPTP administration, producing a clinical condition consistent with 'end-stage PD' in a few days [9]. Still, brain imaging and neuropathological data suggest that, following the acute phase of neuronal death, SNpc neurons continue to succumb at a much lower rate for many years after MPTP exposure [10,11]. From a neuropathological standpoint, MPTP administration causes damage to the dopaminergic pathways identical to that seen in PD [12] with a resemblance that goes beyond the degeneration of SNpc dopaminergic neurons. For instance, like PD, MPTP causes a greater loss of dopaminergic neurons in the SNpc than in the ventral tegmental area [13,14] and a greater degeneration of dopaminergic nerve terminals in the putamen than in the caudate nucleus, at least in monkeys treated with low dose of MPTP [15], but apparently not in acutely intoxicated humans [16]. On the other hand, two typical neuropathologic features of PD have, until now, been lacking in the MPTP model. First, except for the SNpc, the other pigmented nuclei such as the locus coeruleus have been spared, according to most published reports. Second, the eosinophilic intraneuronal inclusions, called Lewy bodies, so characteristic of PD, have thus far not been convincingly observed in MPTP-induced parkinsonism [17]. Interestingly, chronic infusion of rotenone, a mitochondrial poison that exhibits a lot of mechanistic similarities of MPTP, does produce intraneuronal inclusion reminiscent of Lewy bodies in the context of SNpc dopaminergic neuronal death in rats [18]. This finding strongly suggests that whether or not Lewy bodies are seen following administration of a toxin such as MPTP may be more related to the timetable of intoxication than to the nature of the toxin. Also worth noting is the fact that post-mortem brain samples from PD patients [19] show a selective defect in the same mitochondrial electron transport chain complex that is affected by MPTP [20,21]. Abnormalities in parameters of oxidative stress in post-mortem PD brain tissue suggest that this disease is caused by an overproduction of reactive oxygen species (ROS) [22], the same highly reactive tissue damaging species that are suspected of being involved in MPTP-induced dopaminergic toxicity in vivo [23–25]. However, despite this impressive resemblance between PD and the MPTP model, MPTP has never been recovered from post-mortem brain samples or body fluids of PD patients. Altogether, these findings are consistent with MPTP not causing PD, but being an excellent experimental model of PD. Accordingly, it can be speculated that elucidating the molecular mechanisms of MPTP should lead to important insights into the pathogenesis and treatment of PD.

3. Mode of action of MPTP

As illustrated in Fig. 1, the metabolism of MPTP is a complex, multistep process [26]. After its systemic administration, MPTP, which is highly lipophilic, rapidly crosses the blood–brain barrier. Once in the brain, the pro-toxin MPTP is metabolized to 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) by the enzyme monoamine oxidase B (MAO-B) within non-dopaminergic cells, and then (probably by spontaneous oxidation) to 1-methyl-4-phenylpyridinium (MPP^+), the active toxic compound. Thereafter, MPP^+ is released (by an unknown mechanism) in the extracellular space. Brain inflow of MPTP, together with its transforma-

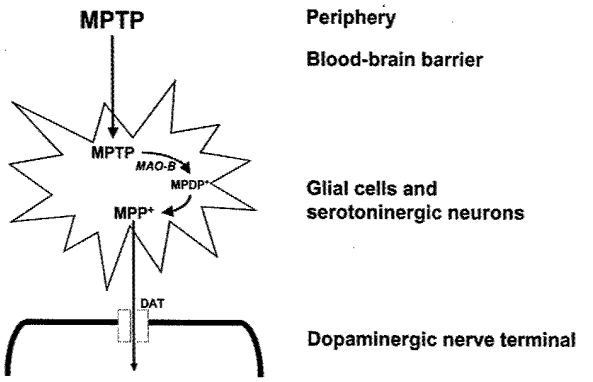

Fig. 1. Schematic representation of MPTP metabolism. After its systemic administration, MPTP crosses the blood–brain barrier. Once in the brain, MPTP is converted to $MPDP^+$ by MAO-B within non-dopaminergic cells, and then to MPP^+ by an unknown mechanism. Thereafter, MPP^+ is released, again by an unknown mechanism, in the extracellular space. From there, MPP^+ is taken up by the DAT and enters into dopaminergic neurons.

tion into MPP^+, determines the amount of MPP^+ available to enter dopaminergic neurons. The next important step in the MPTP neurotoxic pathway is the mandatory entry of MPP^+ into dopaminergic neurons. Since MPP^+ is a polar molecule, unlike its precursor MPTP, it cannot freely enter cells, but depends on the plasma membrane carriers to gain access to dopaminergic neurons. MPP^+ has a high affinity for plasma membrane dopamine transporter (DAT) [27], as well as for norepinephrine and serotonin transporters. The obligatory character of this step in the MPTP neurotoxic process is demonstrated by the fact that blockade of DAT by specific antagonists such as mazindol [28] or ablation of DAT gene in mutant mice [29] completely prevents MPTP-induced toxicity. Conversely, transgenic mice with increased brain DAT expression are more sensitive to MPTP [30].

Once inside dopaminergic neurons, MPP^+ can follow at least three routes: (i) it can bind to the vesicular monoamine transporters (VMAT) which will translocate MPP^+ into synaptosomal vesicles [31]; (ii) it can be concentrated by an active process within the mitochondria [32]; and (iii) it can remain in the cytosol and interact with different cytosolic enzymes [33]. The fraction of MPP^+ destined to each of these routes is probably a function of MPP^+ intracellular concentration and affinity for VMAT, mitochondria carriers, and cytosolic enzymes. The importance of the vesicular sequestration of MPP^+ is demonstrated by the fact that cells transfected to express greater density of VMAT are converted from MPP^+-sensitive to MPP^+-resistant cells [31]. Conversely, we demonstrated that mutant mice with 50% lower VMAT expression are significantly more sensitive to MPTP-induced dopaminergic neurotoxicity compared to their wild-type littermates [34]. These findings indicate that there is a clear inverse relationship between the capacity of MPP^+ sequestration (i.e. VMAT density) and the magnitude of MPTP neurotoxicity.

Inside dopaminergic neurons, MPP^+ can also be concentrated by an active process within the mitochondria (Fig. 2) [32], where it impairs mitochondrial respiration by inhibiting complex I of the electron transport chain [35,36] through its binding at or near the same site as the mitochondrial poison rotenone [37,38]. The binding of MPP^+ to complex I, by interrupting the flow of electrons, leads to a deficit in ATP formation. It appears, however, that complex I activity should be reduced >70% to cause severe ATP depletion [39] and that, in contrast to in vitro, in vivo MPTP causes only a transient 20% reduction in mouse striatal and midbrain ATP levels [40]. This raises the question as to whether MPP^+-related ATP deficit can be the sole factor underlying MPTP-induced dopaminergic neuronal death. Another consequence of complex I inhibition by MPP^+ is an increased production of ROS, especially of superoxide [41], that can react with nitric oxide (NO) produced in non-dopaminergic cells to form peroxynitrite, another potent oxidant. Although compelling evidence supports the notion that the two main mediators of MPTP-toxicity, at least shortly after the toxin administration, are energy crisis and oxidative stress [42,43], more recently it has become apparent that MPP^+-induced mitochondrial dysfunction is also associated with the activation of specific factors of the apoptotic machinery.

In this review, we will address each of these deleterious molecular pathways activated by MPP^+, including some that are not illustrated in Fig. 2, such as the implication of dopamine (DA) autooxidation and the role of the pre-synaptic protein α-synuclein.

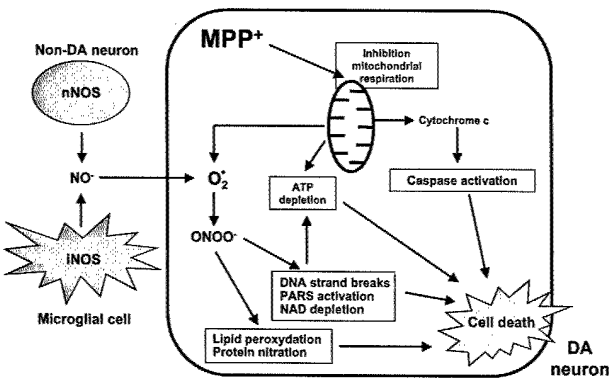

Fig. 2. Mechanisms of MPTP neurotoxicity. Within dopaminergic neurons, MPP^+ inhibits enzymes in the mitochondrial electron transport chain, resulting in ATP deficit and increased 'leakage' of superoxide (O_2) from the respiratory chain. Superoxide remains in the cell in which it is produced. On the other hand, NO, which is produced by nNOS and iNOS outside dopaminergic neurons, is membrane-permeable and can diffuse into neighboring neurons. If the neighboring cell has elevated levels of superoxide, then there is an increased probability of superoxide reacting with NO to form peroxynitrite, which can damage lipids, proteins, and DNA. Damaged DNA stimulates PARS activity, which further depletes ATP stores. On the other hand, MPP^+ may induce the release of cytochrome c from the mitochondria to the cytosol where it initiates a cascade of caspase activation.

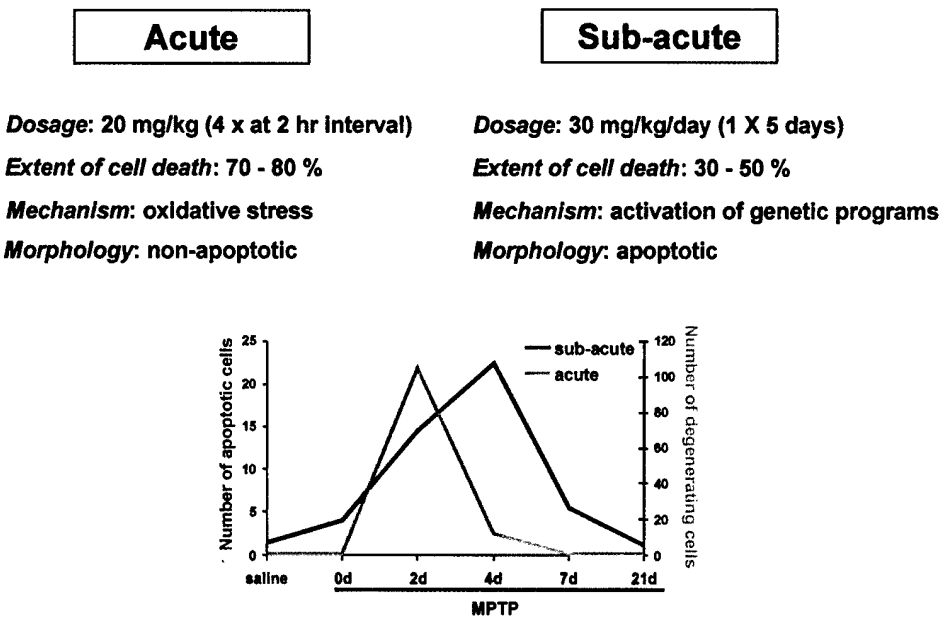

Fig. 3. Main regimens of MPTP administration in mice.

4. Regimens of MPTP intoxication

In the recent years, two different regimens of MPTP administration have been widely used in mice (Fig. 3). The first one, called 'acute,' consists of four intraperitoneal injections of MPTP (20 mg/kg) in saline at 2 h intervals within a single day. This regimen of MPTP administration produces a 70–80% loss of SNpc dopaminergic cells by a mechanism mainly involving oxidative stress, which is associated with a non-apoptotic morphology of cell death [44]. The second regimen of MPTP administration, called 'sub-acute,' consists of one intraperitoneal injection of MPTP per day (30 mg/kg/day) for 5 consecutive days. The extent of SNpc dopaminergic cell loss reached with this regimen is about 30–50%, by a mechanism mainly involving apoptosis [45,46]. Indeed, this specific regimen leads to a dopaminergic cell death with morphological features of shrinkage of cell body, chromatin condensation, and the presence of distinct, round, well defined chromatin clumps, all of which are consistent with those neurons being apoptotic [47]. For the acute regimen, cell death peaks at day 2 after MPTP intoxication, as evidenced by assessing the number of degenerating silver-positive cells in the SNpc, and the lesion is stabilized by day 7 [44]. For the sub-acute regimen, the peak of cell death appears at day 4 after the last MPTP injection, as assessed by counting the number of apoptotic cells in the SN, and the lesion progressively stabilizes by day 21 [46]. Traditionally, the acute regimen has been used mainly to address questions related to the involvement of oxidative stress in MPTP-induced cell death, whereas the sub-acute regimen has been used to study the implication of the molecular pathways of apoptosis in MPTP-induced dopaminergic neurodegeneration. It remains to be determined, however, if there is an overlap of the molecular mechanisms induced by these two different regimens of MPTP intoxication.

5. MPTP, superoxide and nitric oxide

The importance of MPP^+-related superoxide production in dopaminergic toxicity process in vivo is demonstrated by the fact that transgenic mice with increased brain activity of copper/zinc superoxide dismutase (SOD1) are significantly more resistant to MPTP-induced dopaminergic toxicity than their non-transgenic littermates [23]. This finding strongly suggests that superoxide radical plays a pivotal role in the MPTP neurotoxic process. However, superoxide is poorly reactive, and it is the general consensus that this radical does not cause serious direct injury [48]. Instead, superoxide is believed to exert many or most of its toxic effects through the generation of other reactive species such as hydroxyl radical, whose oxidative properties can ultimately kill cells [48]. For instance, superoxide facilitates hydroxyl radical production by hydrogen peroxide and transitional metals such as iron (i.e. Fenton reaction) [48]. Although this reaction can readily take place in vitro, its occurrence in vivo is subordinate to such factors as low pH [49]. Despite this unfavorable pH constraint, MPTP does stimulate the formation of hydroxyl radicals in vivo, as evidenced by the increase in the hydroxyl radical-dependent conversion of salicylate into 2,3- and 2,5-dihydroxy-benzoates [24,50]. Still, there is no convincing demonstration that the produced hydroxyl radical actually causes oxidative damage in the MPTP model [51].

Superoxide can also react with NO to produce peroxyni-

trite (Fig. 2), another potent oxidant [52]. At physiological pH and in aqueous milieu, this reaction proceeds five times faster than the decomposition of superoxide by SOD [53]. The intracellular concentration of SOD1 is estimated at 10–40 μM [54]. Thus, NO concentration has to be ~10 μM for peroxynitrite formation to be competitive, which is not unrealistic as NO production at the cellular level is estimated at 1–10 μM [52]. The situation is different, however, for superoxide, whose basal intracellular concentration is low [55]. Thus, under normal conditions, superoxide is limiting, and it is likely that minimal peroxynitrite formation occurs. Conversely, in pathological conditions, should superoxide concentrations increase, as in response to MPTP administration, formation of appreciable amounts of peroxynitrite is expected. In light of this and of our previous work on superoxide [23], we [56] and others [24,25] have assessed the role of NO in the MPTP neurotoxic process. These studies show that inhibition of NO synthase (NOS) attenuates, in a dose-dependent fashion, MPTP-induced striatal dopaminergic loss in mice [24,56]. We also demonstrate that 7-nitroindazole (7-NI), a compound that inhibits NOS activity without significant cardiovascular effects in mice [57], is profoundly neuroprotective against MPTP-induced SNpc dopaminergic neuronal death [56]. The protective effect of the NOS antagonist 7-NI against MPTP-induced striatal and SNpc dopaminergic damage was subsequently demonstrated in monkeys [25]. Because 7-NI also blocks MAO-B [58], at least part of the reported protection must be due to that effect [59]. The neuroprotection of the pharmacological inhibition of NOS in the mouse MPTP model has been subsequently confirmed thanks to the use of new and 'cleaner' NOS antagonist [60].

However, NOS which produces NO, has, thus far, not been identified inside dopaminergic neurons in rodents; although this needs to be confirmed, low levels of NOS might be present in dopaminergic neurons in humans [61]. In contrast to their lack of NOS, at least in rodents, dopaminergic structures are surrounded by NOS-containing fibers and cell bodies in the striatum, and, to a much lesser extent, in the SNpc [61,62]. Because NO is uncharged [63], it is able to travel away from its site of synthesis and inflict remote cellular damage without the need for any export mechanisms. It is suggested that NO, which is highly diffusible, can travel in random directions up to 150–300 μm during the 5–15 s that correspond to its estimated half-life in physiological aqueous conditions [63]. Although this modeling may depart from the actual *in vivo* situation encountered by a molecule of NO, it gives credence to the hypothesis that NO can cover a distance several times greater than the diameter of a dopaminergic neuron. We are thus speculating that the NO production involved in MPTP toxicity takes place in non-dopaminergic cells present in the vicinity of dopaminergic structures.

Another question pertinent to the origin of NO in the MPTP model is which isoforms of NOS are primarily involved in this process. To date, three distinct NOS isoenzymes have been purified and molecularly cloned: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). Since all three isoforms of NOS have been identified in the brain, each of these can individually or in combination be involved in the production of NO used in MPTP neurotoxic process.

Neuronal NOS is the predominant isoform of NOS in the brain. Its catalytic activity and protein are identifiable throughout the brain [61,64]. Relevant to MPTP, nNOS is present in the striatum within intrinsic medium-sized neurons co-localizing somatostatin and neuropeptide Y [65]. In the midbrain, nNOS is found in cholinergic neurons and within serotoninergic fibers [62,65]. Thus, both by its abundance and its localization, nNOS appears to be an excellent candidate for producing NO for MPTP. In agreement with this is our demonstration that mutant mice deficient in nNOS are partially protected against MPTP-induced striatal dopaminergic toxicity [56]. The finding that mice are better protected by the NOS antagonist 7-NI than by the lack of nNOS expression suggests that, although nNOS is important, it may not be the sole isoform of NOS that is involved in this neurotoxic process.

In the normal brain, iNOS is not detectable [66] nor is it minimally expressed [67]. However, under pathological conditions, iNOS expression can significantly increase in activated astrocytes as well as in other cells such as microglia [68] and invading macrophages. Consistent with this, we have found that, early in the course of MPTP-induced dopaminergic neuron degeneration, there is an increase in midbrain iNOS activity within glial cells [69]. Changes in iNOS activity are already substantial 24 h after MPTP administration, which precedes the peak of dopaminergic neurodegeneration [44]. Therefore, NO derived from iNOS is likely minimal in normal brains, but may become increasingly substantial as MPTP-induced dopaminergic neurodegeneration progresses. Accordingly, iNOS may not play a significant role in the initiation of the MPTP toxic process, but may amplify it and assure its propagation by fueling dopaminergic neurons with increasing amounts of NO. In agreement with this, we have shown that iNOS-deficient animals are more resistant to the neurotoxic effect of MPTP than their wild-type littermates [69].

The third isoform of NOS (eNOS) is highly expressed in the endothelium of blood vessels, including those in the SNpc in close proximity to dopaminergic cells. However, in our experience, it appears that ablation of eNOS has no bearing on MPTP-induced neurotoxicity.

6. MPTP and tyrosine nitration

In light of the above, it appears that, since they are weak oxidants, neither superoxide nor NO is, by itself, sufficiently damaging to participate directly in the MPTP toxic process. In contrast, we have also presented above different arguments supporting peroxynitrite in this role. The versatility

of peroxynitrite as an oxidant is impressive [70,71]. For instance, an important aspect of peroxynitrite's deleterious action is the oxidation of phenolic rings in proteins, and, in particular, of tyrosine residues [72], to form nitrotyrosine as the most important product [73]. As such, detection and quantification of nitrotyrosine are important indirect evidence that peroxynitrite is involved in a pathological process. Relevant to the participation of peroxynitrite in the MPTP model, it has been demonstrated that MPTP significantly increases striatal levels of both free and protein-bound nitrotyrosine in mice [24,51]. Notably, stereotaxic injection of free nitrotyrosine causes striatal neurodegeneration in vivo [74].

Aside from its role as a marker, nitrotyrosine can be a harmful modification as it can inactivate enzymes and receptors that depend on tyrosine residues for their activity [75,76] and prevent phosphorylation of tyrosine residues important for signal transduction [77,78]. This described cascade of events appears quite relevant to MPTP's mode of action as we have demonstrated that, following MPTP administration to mice, not only do both striatal and midbrain levels of nitrotyrosine increase in a time-dependent fashion, but tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, becomes inactivated via a process that involves nitration of its tyrosine residues [79].

7. MPTP, DNA damage and poly(ADP-ribose) polymerase

Thus far, the lion's share of attention has been given to the effects on proteins of reactive species produced after MPTP administration. However, as stated above, most of the reactive species, like peroxynitrite, that may be implicated in the MPTP model, can damage, through oxidative processes, many vital cellular elements other than proteins [48]. Among these, DNA is of unique importance, because it is the repository for genetic information and is present in single copies. Oxidants like peroxynitrite can cause a range of DNA damage [48]. For example, DNA exposed to peroxynitrite produces 8-hydroxyguanine and 8-hydroxydeoxyguanosine, two modifications whose levels seem increased in midbrain of post-mortem PD brains compared to normal controls [80,81]. Because peroxynitrite can nitrate the aromatic group, it can also stimulate the formation of 8-nitrodeoxyguanosine [82]. Finally, intact cells exposed to peroxynitrite exhibit a dose-dependent increase in DNA single strand breakage [83], which is another important type of DNA alteration. In light of the proposed oxidant species involved in MPTP neurotoxicity, all of the aforementioned DNA modifications can possibly occur in this model, as well as in PD. However, despite the potential pathological role of DNA damage, to date we are not aware of any published study on this process in the MPTP model. Nevertheless, we have preliminary data indicating that MPTP does indeed cause conspicuous DNA damage such as strand breaks in SNpc neurons of MPTP-treated mice (S. Przedborski and M.F. Chesselet, personal observation). Although all of the mentioned modifications are potentially mutagenic and thus likely harmful, strand breakage is especially evil because of its link to the enzyme poly(ADP-ribose) polymerase (PARP). Indeed, DNA single strand breakage is an obligatory trigger of the activation of PARP, a phenomenon that we believe, for the reasons that follow, to be a major factor in the overall MPTP-induced cascade of deleterious events. Thus far, the actual functions of PARP remain uncertain, and data obtained with cell-free systems and cells from PARP knockout mice suggest that, contrary to the common belief, PARP would not have a direct role in DNA repair mechanisms [84,85]. On the other hand, it is clear that the activation of PARP results in the cleavage of NAD^+ into ADP-ribose and nicotinamide, both in vitro and in vivo [84,85]. In turn, PARP covalently attaches ADP-ribose to diverse proteins, including nuclear proteins, histones, and PARP itself. PARP then extends the initial ADP-ribose groups into a nucleic acid group-like polymer, poly(ADP-ribose). It is, therefore, manifest that PARP activation, by synthesizing poly(ADP-ribose) polymer, can rapidly deplete intracellular stores of NAD^+ which may impair glycolysis and mitochondrial electron transport chain activities, and, consequently, ATP formation [84,85]. This PARP-dependent cascade of events could play a critical role in the demise of the SNpc dopaminergic neurons as suggested by *in vitro* and *in vivo* data [82,86,87]. This scenario may be even more significant if, as in the case of the MPTP model, the production of ATP in SNpc dopaminergic neurons is already compromised due to the inhibition of the mitochondrial complex I by MPP^+ [40]. In favor of the importance of PARP activation in the MPTP acute neurotoxic process in vivo is our demonstration that PARP is intensely activated following MPTP administration and that mutant mice deficient in PARP are more resistant to MPTP-induced dopaminergic neuronal death [88].

8. MPTP and inflammation

The loss of dopaminergic neurons in the MPTP mouse model and in PD is associated with a glial response composed mainly of activated microglial cells and, to a lesser extent, of reactive astrocytes [89]. This glial response in the SNpc is fairly similar between humans with PD and those intoxicated by MPTP, although a more significant astrocytic reaction is seen in the latter [11]. From a neuropathological standpoint, microglial activation is indicative of an active, ongoing process of cell death. While the presence of activated microglia in PD is consistent with the fact that PD is a progressive condition, their demonstration in postmortem samples from MPTP-intoxicated individuals who came to autopsy several decades after being exposed to the toxin [11], challenges the notion that MPTP produces a 'hit-and-run' kind of damage. Conver-

sely, this important observation [11] suggests that a single acute insult to the SNpc by MPTP could set in motion a self-sustained cascade of events with long-lasting deleterious effects. Looking at mice injected with MPTP and killed at different time points thereafter, it appears that the time course of reactive astrocyte formation parallels that of dopaminergic structure destruction in both the striatum and the SNpc, and that GFAP expression remains up-regulated even after the main wave of neuronal death has passed [69,90,91]. These findings suggest that, in the MPTP mouse model [92], the astrocytic reaction is secondary to the death of neurons and not the reverse. This is supported by the demonstration that blockade of MPP^+ uptake into dopaminergic neurons completely prevents not only SNpc dopaminergic neuronal death but also GFAP up-regulation [93]. Remarkably, activation of microglial cells, which is also quite strong in the MPTP mouse model [69,90,91,94], occurs much earlier than that of astrocytes and, more importantly, reaches a maximum before the peak of dopaminergic neurodegeneration [69]. In light of the MPTP data presented above, it can be surmised that the response of both astrocytes and microglial cells in the SNpc clearly occurs within a timeframe allowing these glial cells to participate in the demise of dopaminergic neurons in the MPTP mouse model and possibly in PD. Activated microglial cells can produce a variety of noxious compounds including ROS, reactive nitrogen species (RNS), pro-inflammatory cytokines and prostaglandins. The latter along with their synthesizing enzymes, such as cyclooxygenase type-2 (Cox-2), have emerged as an important determinant of cytotoxicity associated with inflammation [95,96]. In the normal brain, Cox-2 is significantly expressed only in specific subsets of forebrain neurons that are primarily glutamatergic in nature [97], which suggests a role for Cox-2 in the postsynaptic signaling of excitatory neurons. However, under pathological conditions, especially those associated with a glial response, Cox-2 expression in the brain can increase significantly, as does the level of its products (e.g. prostaglandin E_2), which are responsible for many of the cytotoxic effects of inflammation. Interestingly, Cox-2 promoter shares many features with iNOS promoter [98] and thus, these two enzymes are often co-expressed in disease states associated with gliosis. Therefore, it is not surprising to find Cox-2 and iNOS expressed in SNpc glial cells of post-mortem PD samples [99]; PGE_2 content is also elevated in SNpc from PD patients [100]. Of relevance to the potential role of prostaglandin in the pathogenesis of PD, is the demonstration that the pharmacological inhibition of both Cox-2 and Cox-1 attenuates MPTP toxicity in mice [101].

9. MPTP and dopamine oxidation

The mitochondria is a well recognized site of ROS production, making this organelle likely instrumental in MPTP-mediated oxidative damage to SNpc dopaminergic neurons. However, for many years the notion that ROS can also emanate from the cytosol of dopaminergic neurons as a result of dopamine autooxidation [102], has been overlooked or underestimate by most investigators in the field. Relevant to this are the demonstrations that MPP^+ induces, both in vivo and in vitro, a massive and rapid outflow of dopamine from intracellular pools [103–108]. This phenomenon raises the possibility that, following MPTP administration, intracellular dopamine oxidation may represent a significant source of ROS of possibly even greater magnitude than that originating from the mitochondrial dysfunction. In support of this view, cultured ventral mesencephalic murine neurons exposed to MPP^+ exhibit a massive displacement of dopamine from synaptic vesicles to the cytosol [109]. By depleting both stored and newly synthesized dopamine using the VMAT blocker reserpine and the TH inhibitor α-methylparatyrosine, MPP^+-induced ROS formation is prevented and cell death attenuated. These results indicate that, in vitro, MPP^+-induced ROS formation may not only originate from the mitochondria but also from dopamine oxidation within the cytosol.

10. MPTP and α-synuclein

The potential role of α-synuclein in the pathophysiology of PD has attracted a great deal of attention since: (i) mutations in the α-synuclein gene have been found to be responsible for a rare familial form of PD that is clinically and pathologically indistinguishable from the most common sporadic form of the disease [110,111]; and (ii) α-synuclein has been identified as a major component of Lewy bodies, one of the pathologic hallmarks of PD [112]. Cytotoxicity of mutant α-synuclein is likely related to the fact that both of the identified point mutations may enhance the propensity of α-synuclein to interact with other intracellular proteins and increase its tendency to aggregate [113–118]. α-Synuclein mutations are not found in sporadic PD, raising the hypothesis that effects similar to those of familial PD-linked α-synuclein mutations may be achieved by oxidative post-translational modifications. In this context, we have shown that wild-type α-synuclein is a selective target for nitration following peroxynitrite exposure of stably transfected HEK293 cells [119]. Moreover, nitration of α-synuclein, but not of β-synuclein or synaptophysin, occurs in the mouse striatum and ventral midbrain following an acute administration of MPTP [119]. This data demonstrate that α-synuclein is a specific target for MPTP-induced oxidative attack, which may disrupt its biophysical properties by tyrosine nitration. According to this hypothesis, it has been shown that nitrated α-synuclein is present in the Lewy bodies of PD and other Lewy body disorders and in the major filamentous building blocks of these inclusions, as well as in the insoluble fractions of the affected brain regions [120].

Even if after MPTP administration in mice there is no

formation of intracellular inclusions, we have shown that α-synuclein is up-regulated and accumulated into the cytosol of dopaminergic neurons after a sub-acute MPTP intoxication to mice [121]. Similar results have been found in MPTP-intoxicated primates [119]. As we will discuss later, the sub-acute regimen of MPTP intoxication in mice induces translocation of cytochrome c from the mitochondria to the cytosol (M. Vila and S. Przedborski, personal observation). In connection with this is the observation cytochrome c induces α-synuclein aggregation in vitro [122,123]. It is thus possible that upon the released cytochrome c in the cytosol of dopaminergic cells, accumulation and subsequent aggregation of α-synuclein occur.

Taken together, these results indicate that α-synuclein is post-translationally modified by oxidative stress after MPTP intoxication, which probably results in structural modifications of this protein that may increase its tendency to aggregate. In this context, it is interesting to note that overexpression of α-synuclein per se does not have a pathological effect on SNpc dopaminergic cells in vivo [124–127]. These results suggest that α-synuclein needs to be post-translationally modified (either by oxidative stress, in sporadic PD, or by a point mutation, in the familial forms of PD with α-synuclein mutations) in order to acquire its pathological properties, likely involving its aggregation and the formation of intracellular inclusions.

11. MPTP and apoptosis

Mounting evidence indicates that highly regulated cell death-associated molecular pathways could participate in the relentless demise of neurons in PD [47]. As mentioned earlier, a sub-acute regimen of MPTP administration in mice induces activation of the apoptotic molecular pathways. The first observation that apoptotic cell death occurs after MPTP administration was made by visualizing clumped chromatin, an indication of apoptosis, by terminal deoxynucleotidyl transferase labeling (TUNEL) and acridine orange staining [45]. Subsequent studies have implicated different molecular factors of the apoptotic pathways in the mechanisms of MPTP neurotoxicity. For example, we demonstrated that the pro-apoptotic protein Bax plays a key role in the MPTP neurotoxic process [46]. Bax is highly expressed in dopaminergic cells of the SNpc, in both mitochondria and cytosol. After a sub-acute MPTP administration, Bax mRNA and protein are strongly upregulated [46,128]. At the same time, anti-apoptotic molecules, such as Bcl-2, are downregulated in the ventral midbrain after MPTP intoxication, with a resulting increased pro-cell death conformation of Bax [46]. The upregulation of Bax is associated with the release of cytochrome c from the mitochondria to the cytosol (M. Vila and S. Przedborski, personal observation) which may well account for the activation of the downstream executioner caspase-3 [129]. The pivotal role of Bax in MPTP-induced neurotoxicity is illustrated by the fact that ablation of Bax in mutant animals attenuates cytochrome c release and caspase-3 activation (M. Vila and S. Przedborski, personal observation), and prevents dopaminergic cell death after MPTP [46]. Consistent with this scenario, overexpression of the anti-apoptotic Bcl-2 also protects dopaminergic cells against MPTP-induced neurodegeneration [130,131]. Similarly, adenovirus-mediated transgene expression of the X-chromosome-linked inhibitor of apoptosis protein (XIAP), a inhibitor of executioner caspases such as caspase-3, also blocks the death of dopaminergic neurons in the SNpc following the administration of MPTP [132,133]. Other caspases are also activated in MPTP-intoxicated mice and in PD patients, such as caspase-8, which is a proximal effector of the tumor necrosis factor receptor family death pathway [134]. Other observations supporting a role of apoptosis in MPTP neurotoxic process include the demonstration of the resistance of mutant mice deficient in p53 [135], a cell cycle control gene involved in programmed cell death, or of mice treated with inhibitors of c-Jun N-terminal kinases [136,137].

12. Conclusions

The current understanding of the MPTP mode of action proposes that MPP^+ causes mitochondrial dysfunction, oxidative stress, energetic failure and activation of genetic programs leading to cell death. Oxidative stress mediated by superoxide and NO leads to damage of proteins, lipids, and DNA, all contributing to major cellular dysfunctions. In addition, DNA damage leads to activation of PARP, which aggravates the already reduced pool of ATP due to the blockade of complex I by MPP^+. This will impair numerous vital cellular reactions that are ATP-dependent. Subsequently, oxidative stress- and energy failure-related damage affect the cell's ability to maintain intracellular potential, leading to the death of the neurons. On the other hand, MPP^+ induces the activation of molecular pathways of apoptosis. The recruitment of the mitochondrial-dependent apoptotic factors seems to play a pivotal role, with caspase-3 as the main executioner of cell death. Finally, 'the two new kids on the block' namely autooxidation of dopamine and accumulation of α-synuclein, appear to also participate in a significant fashion in the MPTP neurotoxic process. Because of the close similarity between PD and the MPTP model, it is likely that a similar cascade of deleterious events underlies the pathogenesis of PD.

Acknowledgements

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18

The Last Decade in Parkinson's Disease Research

Basic Sciences

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Parkinson's disease (PD) is a common neurodegenerative disorder whose cardinal features include tremor, slowness of movement, stiffness, and poor balance (1). Most, if not all, of these disabling symptoms are secondary to a profound reduction in striatal dopamine content, caused by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) and of their projecting nerve fibers in the striatum (2,3). Although several approved drugs do alleviate PD symptoms, their chronic use is often associated with debilitating side effects (4), and none seems to dampen the progression of the disease. Moreover, the development of effective preventive or protective therapies is impeded by our limited knowledge of the cause (i.e., etiology) and mechanisms (i.e., pathogenesis) by which dopaminergic neurons die in PD.

Although neither the etiology nor the pathogenesis of PD has yet been elucidated, this last decade has witnessed an explosion of invaluable research, which unquestionably has provided critical insights into our current understanding of this illness. Accordingly, in this chapter, we give an overview of what we believe are the key findings of the past 10 years in this area. We also try to place each of these findings within the context of what appears to be, at least to date, the direction in which the field of PD research seems to be evolving. One caveat of our approach resides in the fact that the goal of this chapter is to provide a "flavor" for the field of PD research rather than a comprehensive review. Therefore, the reader must be aware that only selected aspects of the research performed in PD are reviewed and discussed. Along this line, the reader should also know that this chapter does not, except incidentally, review the large core of research dealing with "symptomatic therapies," whether the approach is pharmacological or surgical, which are discussed elsewhere in this book.

ETIOLOGY OF PD

If the goal is to prevent PD and to diagnose it before any actual neurodegeneration occurs, then we must unravel the etiology of this disorder. For many years, the two main hypotheses for the etiology of PD that have prevailed have been the toxic and the genetic hypotheses. As we will see, there is supportive evidence for both hypotheses, and neither one is exclusive of the other.

Toxic Hypothesis

According to this hypothesis, it is proposed that a deleterious compound may be present in our environment, even in low amounts, and

that over time it may accumulate in our organism and ultimately reach a threshold level that will cause it to unleash its damaging properties against the dopaminergic system. A significant support for an "exogenous or environmental toxin" has been provided by the discovery that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) can cause a parkinsonian syndrome in humans almost identical to PD (5). Also relevant is the fact that 1-methyl-4-phenylpyridimium (MPP^+), MPTP's active metabolite, has been extensively used as an herbicide in many countries around the world, as has paraquat, which has a striking structural similarity to MPP^+. Furthermore, a rural environment has often been found to be associated with an elevated risk of PD (6). In keeping with this, factors such as herbicides, pesticides, and well water have all been incriminated in the occurrence of PD (6). To date, it remains, however, that despite all of these supportive observations, no actual compound, related to the MPTP family or not, has been unequivocally linked to the development of PD. Alternatively, it has also been proposed that perhaps the putative parkinsonian toxin is not exogenous but rather is produced by our own metabolism, giving rise to the "endogenous toxin" hypothesis of PD (7). According to this model, a noxious compound would be produced in response to either a defective or a variant metabolic pathway. In keeping with this view is the suggestion that patients harboring specific polymorphisms in the gene encoding for the cytochrome P450 may be at greater risk of developing young-onset PD (8). Furthermore, several isoquinoline derivatives, which can kill dopaminergic neurons, have been recovered from the brains of PD patients and thus are regarded by several experts as potential endogenous parkinsonian neurotoxins (9). Among these, tetrahydroisoquinoline (TIQ), 1-benzyl-TIQ, and *(R)*-1,2-dimethyl-5,6-dihydroxy-TIQ have the most potent neurotoxic effects (9).

Genetic Hypothesis

During the last decade, there has been a clear waning and waxing of enthusiasm for the role of genetics in the etiology of PD. However, during the past few years there has been an upsurge of interest in the genetics of PD, triggered by several breakthroughs obtained in familial forms of parkinsonism. For instance, point mutations in the α-synuclein gene, located on chromosome 4, have been found to cause an autosomal dominant parkinsonian syndrome (10,11). The two missense mutations identified thus far result in a single amino acid substitution in α-synuclein protein, that is, an alanine being replaced by a hydrophobic residue threonine at position 53 and by proline at position 30. Since the discovery of these mutations, data have been accumulated suggesting that both mutations may alter α-synuclein's normal intracellular distribution, enhance α-synuclein's propensity to interact with other intracellular proteins, and increase α-synuclein's disposition to aggregate and consequently to form intraneuronal inclusions (12–16). However, thus far, efforts to identify α-synuclein mutations in sporadic PD have failed (17–19). On the other hand, in sporadic PD, α-synuclein has been demonstrated to be a major component of the intraneuronal inclusions Lewy bodies, which are a pathologic hallmark of the disease (20,21). In addition, we have recently demonstrated that α-synuclein is up-regulated in dopaminergic neurons of the SNc after MPTP administration to mice (22). However, the normal function of this protein and its implications for the pathogenesis of PD remain to be determined.

The gene for an autosomal recessive form of juvenile parkinsonism has also recently been identified and encoded for a protein called parkin (23). More recently, a susceptibility locus for PD has been mapped to chromosome 2p13 (24), and a point mutation in the gene encoding for a key enzyme of the ubiquitin pathway has been identified in a family with parkinsonism (25). In light of all of these discoveries, we can conclude that there is increasing evidence that genetic factors might play a role in PD. However, these studies also show quite clearly that only a small number of the multigenerational fam

ked in the cited investigations have con- pathology of PD, and most of the af- family members exhibit atypical fea- such as an onset at younger age, rapid se, and often dementia. Moreover, the of inheritance of the parkinsonism hin these families is highly variable, rang- from autosomal dominant to autosomal essive and even the possibility of being ternally transmitted (26), raising the poten- lity of a genetic defect within the mito- hondrial genome. Because, to date, none of identified mutations found in familial PD has been identified in sporadic PD, it is likely that these genetic alterations may account, at most, for a very small fraction of PD patients. Nevertheless, these findings remain extremely exciting, as they raise the prospect that by elucidating the actual mechanisms by which these genetic defects produce the demise of dopaminergic neurons in familial forms of PD, they may well shed light into the etiology and pathogenesis of sporadic PD.

In our opinion, one of the most damaging arguments against a pivotal genetic component in the etiology of PD is provided by the lack of significant concordance in monozygotic twins with classical PD (27). It remains plausible that genetics may play a critical role, not as a unique etiologic factor but rather within a multifactorial cascade such as through an interaction between genetic and toxic mechanisms. This view is supported by the demonstration that individuals carrying a specific mitochondrial mutation will develop deafness only on exposure to aminoglucoside (28). Conversely, it is also relevant to point out that, among the cohort of individuals who were intoxicated with MPTP, only a fraction developed parkinsonism (Dr. J. W. Langston, *personal communication*). These two examples emphasize the potential importance of the interaction between genetic and toxic factors.

PATHOGENESIS OF PD

In spite of the above-described efforts in identifying the etiology of PD, affected patients to date are diagnosed only when the symptoms of the disease have already appeared. Therefore, it is important to identify the mechanisms involved in the cellular death in order to halt or slow down the progression of the disease once it is already established.

In an attempt to unravel the mechanisms implicated in the neurodegenerative process in PD, a large number of presumed pathogenic factors have been put forward, including dopamine metabolism, mitochondrial dysfunction, free radicals, cell death by apoptosis, excitotoxicity, defect in trophic factors, and many others. Some of these are discussed below.

Dopamine Metabolism

Dopamine is the neurotransmitter of the SNc neurons controlling normal motor function. It seems, however, that dopamine is not essential for the normal development of the SNc system, as observed in mice lacking tyrosine hydroxylase (TH), the enzyme catalyzing the first and rate-limiting step of catecholamine biosynthesis. It is worthy to note that although these mutant mice have almost no dopamine production, they show a normal cytoarchitecture of the SNc dopaminergic system (29,30) as evidenced by immunostaining for DOPA decarboxylase, another enzyme in this synthetic pathway. Interestingly, between 2% and 22% of wild-type catecholamine concentrations are found in the brains of these mutant (31) mice, likely as the result of alternative synthetic pathways such as that involving tyrosinase, another enzyme that converts tyrosine to L-DOPA but that does so during melanin synthesis.

In the mature brain, although it is clear from PD that dopamine is essential for motor control, it has been frequently suggested that dopamine at the same time may exert deleterious effects that may participate in the progression of the disease. Evidence in support of this view is still lacking *in vivo* in that there is no definitive demonstration that individuals who erroneously receive high doses of dopamine precursor, L-DOPA, for a pro-

longed period fare worse because of the drug. Similarly, the toxic effect of dopamine/L-DOPA has not been observed in studies using animals with intact nigrostriatal pathway. More confusing is the situation in rats with moderate SNc damage produced by the neurotoxin 6-hydroxydopamine, which generated contradictory results (32,33). The issue of dopamine-mediated toxicity is more compelling when one looks at the large core of *in vitro* studies dealing with this question. For instance, it has been shown that 200 μM of L-DOPA can cause a 50% reduction in the number of dopaminergic neurons in postnatal midbrain cultures (34). This toxic effect seems to be mediated by the production of free radicals because it is prevented by the overexpression of copper/zinc superoxide dismutase (SOD1) (34), a key free radical–scavenging enzyme.

Another intriguing aspect related to the dopamine metabolism that has not yet been solved concerns the link between the vulnerability of SNc dopaminergic neurons and the prominent content in black pigmentation called neuromelanin in these neurons (35). It has been reported that (a) the dopamine-containing cell groups of the normal human midbrain differ markedly from each other in the percentage of neuromelanin-pigmented neurons they contain; (b) the estimated cell loss in these cell groups in PD is directly correlated with the percentage of neuromelanin-pigmented neurons normally present in them; and (c) within each cell group in PD brains, there is greater relative sparing of nonpigmented than of neuromelanin-pigmented neurons (36). These results suggest a selective vulnerability of the neuromelanin-pigmented subpopulation of mesencephalic dopaminergic neurons in PD. To date, however, the role of neuromelanin within dopaminergic cells and its origin are not known. A new insight in this field comes from the observation that, in postnatal midbrain cultures exposed to low doses of L-DOPA, dopaminergic neurons accumulate a black pigment with the same characteristics as neuromelanin (36a). This finding indicates that the formation of neuromelanin is clearly related to the presence of L-DOPA/dopamine, and thus, this *in vitro* cellular system may represent a new tool with which to study the actual role played by neuromelanin in the neurodegenerative process. Another unresolved issue inherent to dopaminergic neuron degeneration in PD is the potential contribution of Lewy bodies in the death of these neurons. Along this line, Dr. Sulzer's group has also found that incubation of monoaminergic clonal PC-12 cells with L-DOPA induces ubiquitinated intracellular inclusions reminiscent of Lewy bodies. This exciting finding may enable us to identify the factors involved in the formation of the Lewy bodies as well as to determine their actual role in the neurodegenerative process.

Dopamine metabolism by monoamine oxidase or by autooxidation leads to the formation of hydrogen peroxide, superoxide radicals, and several reactive quinones and semiquinones that could contribute to the heightened state of oxidative stress in PD (37). Neuromelanin within dopaminergic neurons can bind ferric iron and reduce it to its reactive ferrous form (35). Taken together, these results show that the SNc, because of its dopamine and neuromelanin content, is a designated target for oxidative attack. This view leads us now to discuss the important questions of oxidative stress and of mitochondrial dysfunction in PD (37).

Oxidative Stress and Mitochondrial Dysfunction

Several lines of evidence suggest that the SNc in PD is the site of an oxidative stress (37). As mentioned above, several powerful oxidants are produced in the course of normal metabolism, including hydrogen peroxide, superoxide, peroxyl and hydroxyl, and even nitric oxide (NO). These molecules may cause cellular damage by reacting with nucleic acids, proteins, lipids, and other molecules. Indeed, in the SNc of parkinsonian patients there is evidence of increased malondialdehyde and hydroperoxidase, which suggests lipid peroxidation, increased carbonyl proteins suggesting oxidized proteins, increased

8-hydroxy-2-deoxyguanosine suggesting DNA damage, elevation of iron levels, increase in γ-glutamyl transpeptidase activity, and diminished reduced glutathione. The possibility that oxidative stress participates in the pathogenesis of PD offers therapeutic strategies based in the use of antioxidant agents in order to provide neuroprotection. These may include free radical scavengers, glutathione-enhancing agents, iron chelators, and drugs that interfere with the oxidative metabolism of dopamine. To date, clinical trials have been performed with vitamin E and deprenyl but have failed to show definite neuroprotection.

One main source of reactive oxygen species is the mitochondria. It is thus relevant to mention that a reduction in the activity of the complex I (NADH-ubiquinone oxidoreductase) of the mitochondrial electron transport chain in PD brains has been reported (38). This defect could subject cells to oxidative attack as well as energy failure. Furthermore, it seems that the mitochondrial defect found in PD is generalized and not confined to the brain, as reduced complex I activity has been reported in platelets from PD patients. In addition, hybrid cells, in which mitochondrial DNA has been destroyed and repopulated with mitochondrial DNA from the platelets of PD patients, reproduce the defect in complex I activity (39). The latter finding suggests that the observed complex I deficit originates from an alteration in the mitochondrial rather than the nuclear genome.

It is of importance to indicate that although mitochondrial dysfunction and oxidative metabolism may well be critical components in the cascade of deleterious events leading to the death of SNc dopaminergic neurons, surprisingly none of the data available to date did address the question as to whether these abnormalities represent a primary or secondary events. Indeed, all of these data are merely circumstantial and correlative observations reported in autopsied brains in which most of the dopaminergic cells have already been destroyed, and thus the mechanistic value of autopsy findings must be taken with a great deal of caution.

Programmed Cell Death

In recent years, there has been growing interest in the manner in which neuronal cells degenerate. In this context, the concept that programmed cell death (PCD) may play a role in the pathogenesis of neurodegenerative disorders has emerged as an important hypothesis. PCD represents an active form of cell death in which intrinsic cellular genetic programs are activated, leading to cellular "suicide." This form of death must be distinguished from the presumed passive cellular death resulting from a noxious effect or harsh environmental factors. PCD is also referred to as apoptosis because apoptosis is probably the most common morphologic type of PCD. However, it is important to mention that apoptosis refers to a specific set of morphologic features and is not the sole and unique morphologically defined form of death encountered in PCD (40). For instance, apoptosis is defined by the association of cell body and nucleus shrinkage, chromatin clump formation, DNA fragmentation, and condensation of cytosol and nucleosol, often with preservation of organelles and phenotypic markers. The question of whether apoptosis occurs in neurodegenerative disorders should not be regarded as an esoteric academic problem but rather as a line of research that can shed light into the pathogenesis of PD as well as open new therapeutic avenues.

It has been reported that apoptosis occurs in the substantia nigra during normal development in rodents (41,42). It has also been demonstrated that this phenomenon is time dependent, paralleling the time-course of synaptogenesis, is modulated by factors derived from the target (and/or postsynaptic neurons), and occurs in dopaminergic neurons *per se* as evidenced by TH immunostaining (41–44). Occurrence of SNc PCD has also been examined in mature brains by studying experimental models of PD. Along this line, it has been reported that intrastriatal injection of 6-hydroxydopamine (6-OHDA) in developing animals results in the induction of apoptosis in SNc dopaminergic neurons (45). This effect

seems to be explained by the destruction of dopaminergic terminals by 6-OHDA, thus interfering with target support, rather than a direct action of the toxin-inducing apoptosis. The ability of intrastriatal 6-OHDA to induce apoptotic death is developmentally dependent, with a major induction of death during the first two postnatal weeks but only a minor effect at later postnatal times. Furthermore, at later postnatal days, 6-OHDA-induced cell death presented two different morphologies, apoptotic and nonapoptotic. This suggests either that the toxin induces cell death by two different mechanisms or that the same fundamental mechanism induces an apoptotic morphology in less mature animals and a nonapoptotic morphology in more mature animals. Studies with MPTP in mice have reported mixed results. We initially reported that acute administration of MPTP, in which the drug was given in four separate doses administered every 2 hours, resulted in a nonapoptotic cell death in the SNc (46). More recently, Tatton and Kish have reported (47) in a chronic model of MPTP administration (30 mg/kg per day for five consecutive days) the occurrence of apoptosis in phenotypically defined dopaminergic neurons. Therefore, PCD plays a role in the MPTP mouse model of PD, depending on the administration schedule of the neurotoxin. Finally, in PD, the situation is more complex in that there is mixed evidence concerning whether apoptotic morphology can be identified in the postmortem PD brains of patients (48). It is important to note that apoptotic cell death seems to take place within a short period of time, making its identification difficult in a chronic degenerative disease, and that the quality of the autopsied material might not allow high-quality morphologic studies to be performed.

The MPTP Mouse Model of PD

The fact that MPTP causes a parkinsonian syndrome was discovered in 1982 when a group of drug addicts in California exhibited a severe and irreversible akinetic rigid syndrome analogous to PD (49). Subsequently, it was found that this syndrome was induced by the self-administration of a synthetic heroin analog whose synthesis had been heavily contaminated by a by-product, MPTP (5). Since then, MPTP has been used extensively as a model of PD (5,50,51). From neuropathologic data, MPTP administration causes damage to the dopaminergic pathways identical to that seen in PD (52). Like PD, MPTP causes a greater loss of dopaminergic neurons in the SNc than in the ventral tegmental area (53,54) and a greater degeneration of dopaminergic nerve terminals in the putamen than in the caudate nucleus (55). On the other hand, the eosinophilic intraneuronal inclusions, Lewy bodies, so characteristic of PD, have thus far not convincingly been observed in MPTP-induced parkinsonism (56). However, MPTP has never been recovered from postmortem brain samples or body fluids of PD patients, consistent not with MPTP causing PD but with its being an excellent experimental model of PD. Accordingly, it can be speculated that elucidating the molecular mechanisms of MPTP should lead to important insights into the pathogenesis and treatment of PD.

The metabolism of MPTP is a complex, multistep process (57). After its systemic administration, MPTP, which is highly lypophilic, rapidly crosses the blood–brain barrier and, once in the brain, this protoxin is metabolized to 1-methyl-4-phenyl-2,3-dihydropyridimium ($MPDP^+$) (by the enzyme monoamine oxidase type B) and then to MPP^+. Thereafter, MPP^+ gains access to dopaminergic neurons by binding to plasma membrane dopamine transporter (DAT) (58). The obligatory character of this step in the MPTP neurotoxic process is demonstrated by the fact that blockade of DAT by specific antagonists such as mazindol (59) or ablation of DAT gene in mutant mice (60) completely prevents MPTP-induced toxicity. Conversely, transgenic mice with increased brain DAT expression are more sensitive to MPTP (61).

Inside dopaminergic neurons, MPP^+ can be concentrated by an active process within the mitochondria (62), where it impairs mitochondrial respiration by inhibiting complex I

of the electron transport chain (63–65). The inhibition of complex I impedes the flow of electrons along the mitochondrial electron transport chain, leading to a deficit in ATP formation. It appears, however, that complex I activity must be reduced by at least 70% to cause severe ATP depletion (66) and that, in contrast to the situation *in vitro, in vivo* MPTP causes only a transient 20% reduction in mouse striatal and midbrain ATP levels (67). Another consequence of complex I inhibition by MPP^+ is an increased production of free radicals, especially of superoxide (68–70). The importance of MPP^+-related superoxide production in the dopaminergic toxicity process *in vivo* is demonstrated by the fact that transgenic mice with increased brain activity of SOD1 are significantly more resistant to MPTP (71). However, superoxide is poorly reactive, and it is the general consensus that this radical does not cause serious direct injury (72). Instead, superoxide is believed to exert many or most of its toxic effects through the generation of other reactive species such as hydroxyl radical, whose oxidative properties can ultimately kill cells (72).

Superoxide can also react with NO to produce peroxynitrite, a potent oxidant (73). In light of this and of our previous work on superoxide (71), we (74) and others (75,76) have assessed the role of NO in the MPTP neurotoxic process. These studies show that inhibition of NO synthase (NOS) by 7-nitroindazole (7-NI), a compound that inhibits NOS activity without significant cardiovascular effects in mice (77), attenuates, in a dose-dependent fashion, MPTP-induced dopaminergic toxicity (74,75). The protective effect of the NOS antagonist 7-NI against MPTP-induced dopaminergic damage was subsequently demonstrated in monkeys (76).

Neuronal NOS (nNOS) is the predominant isoform of NOS in the brain (78,79). Both by its abundance and its localization, nNOS appears to be an excellent candidate for producing NO for MPTP; in agreement with this possibility is our demonstration that mutant mice deficient in nNOS are partially protected against MPTP (74). The finding that mice are better protected by the NOS antagonist 7-NI than by the lack of nNOS expression suggests that isoforms other than nNOS may also be involved in MPTP neurotoxic process. Consistent with this view, it should be mentioned that inducible NOS (iNOS), which is not or is only minimally expressed in normal brains (80,81), is dramatically up-regulated after injury including that produced by MPTP (82). Indeed, early in the course of MPTP-induced dopaminergic neuron degeneration, there is an increase in midbrain iNOS activity within the robust glial reactions that occur in the SNc following the administration of this toxin (82). Consistent with the important role of iNOS in the MPTP neurotoxic process is our demonstration that mutant mice deficient in iNOS are more resistant to MPTP (82).

Among the various forms of damage produced by peroxynitrite, the presumed culprit in MPTP-mediated toxicity, is the oxidation of phenolic rings in proteins, and in particular of tyrosine residues (83), to form nitrotyrosine as the most important product (84). Thus, detection and quantification of nitrotyrosine provide important indirect evidence that peroxynitrite is involved in a pathologic process. Relevant to the participation of peroxynitrite in the MPTP model, it has been demonstrated that MPTP significantly increases striatal levels of nitrotyrosine in mice (75,85). Aside from its role as a marker, nitrotyrosine can be a harmful modification, as it can inactivate enzymes and receptors that depend on tyrosine residues for their activity (86,87) and prevent phosphorylation of tyrosine residues important for signal transduction (88,89). This cascade of events appears quite relevant to MPTP's mode of action, as we have demonstrated that, following MPTP administration to mice, TH becomes inactivated by tyrosine nitration (90). Furthermore, peroxynitrite can damage, through oxidative processes, many vital cellular elements other than proteins (72). Among these, DNA is of unique importance because it is the repository for genetic information and is present in a single copy. Oxidants such as peroxynitrite can cause a

range of DNA damage (72), which can possibly occur in the MPTP model as well as in PD. Indeed, our preliminary data generated in collaboration with Dr. M. F. Chesselet (Department of Neurology, UCLA) indicate that MPTP causes conspicuous DNA damage such as strand breaks in SNc neurons in mice.

CONCLUSION

This summary of a quite prolific decade has attempted to outline the findings and the direction of PD research, which we believe should lay the groundwork for the research that will take place during the coming new millennium. As illustrated above, unquestionable progress has been made toward discovering the etiology and the pathogenesis of the disease. In light of this, and although much work is still before us, we should enter this new era with significant hope and enthusiasm for finding a cure for PD.

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The Role of Nitric Oxide in Parkinson's Disease

Serge Przedborski and Ted M. Dawson

1. Introduction

Neurodegenerative disorders are adult-onset disabling neurologic conditions such as Parkinson's disease (PD) in which specific subsets of brain neurons are dying. Without exception, the frequency of these disorders is increasing dramatically as the proportion of elderly in our society grows. The search for efficacious therapies, if not to prevent, at least to slow down or halt the progression of these diseases, is of major public health importance. However, this goal can only be achieved through a better understanding of the causes and mechanisms by which neurons die in these degenerative disorders. We review here the question of nitric oxide (NO), a small and ubiquitous molecule believed to be a pivotal element in the cascade of deleterious events underlying neurodegeneration in PD. Since NO synthases (NOS) are the only known enzymes that produce NO, in this chapter particular attention is paid to the anatomic distribution and catalytic activity of these enzymes in the brain. We also provide several experimental protocols commonly used for quantitative and qualitative studies of NOS.

1.1. Nitric Oxide

NO has emerged as a protean biologic effector molecule that acts as an intercellular messenger molecule in the nervous system, regulating vascular tone and blood pressure, and controlling platelet activation, and when synthesized in high amounts by activated macrophages, it is an antimicrobial and antitumor molecule. Although NO is a gas in its native state, in most biologic systems it acts as a dissolved nonelectrolyte. The only exception is in the lung and in paranasal sinus air, where it is present in the gaseous phase. NO is synthesized by one of the three distinct NOS isoforms isolated to date. Depending

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on the site of production, the amount of NO produced, and the targets within the local environment, NO can have many diverse functions. The identification of NO as a neurotransmitter has refined our conventional understanding of how neurons communicate, since, in contrast to classical neurotransmitters, NO is not stored in synaptic vesicles, is not released by exocytosis, and does not mediate its actions by binding to cell surface receptors. In the nervous system, NO has a dual role as a physiologic messenger and as a mediator of lethal processes in a variety of neurodegenerative disorders and toxic insults to the nervous system.

1.2. Parkinson's Disease

The cardinal clinical features of PD include tremor, stiffness, and slowness of movement, all of which are attributed to the dramatic loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) *(1)*. Its prevalence has been estimated at approx 1,000,000 in North America, with approx 50,000 newly affected individuals each year *(1)*. The most potent treatment for PD remains the administration of a precursor of dopamine, L-dopa, which, by replenishing the brain with dopamine, alleviates almost all PD symptoms. However, chronic administration of L-dopa often causes motor and psychiatric side effects that may be as debilitating as PD itself *(2)*. Furthermore, there is no supportive evidence that L-dopa therapy impedes the progressive death of SNpc dopaminergic neurons. Therefore, without undermining the importance of L-dopa therapy in PD, it remains essential to elucidate the cascade of events that underlie the neurodegenerative process. To this end and in light of the rarity of available postmortem brain samples from PD patients, many investigators, including ourselves, have focused their research efforts on experimental models of PD such as the one produced by the parkinsonian toxin l-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Consequently, most of the currently available data regarding NO in PD derive from studies carried out in the MPTP model and not in PD *per se*.

1.3. The MPTP Model of Parkinson's Disease

MPTP is a byproduct of the chemical synthesis of a meperidine analog with potent heroin-like effects. MPTP can induce a parkinsonian syndrome in humans almost indistinguishable from PD *(3)*. It was recognized as a neurotoxin in early 1982, when several young drug addicts mysteriously developed a syndrome similar to PD after the intravenous use of street preparations of meperidine analogs contaminated with MPTP *(4)*. Since the discovery that MPTP causes parkinsonism in human and nonhuman primates as well as in various other mammalian species, it has been used extensively as a model of PD *(3,5,6)*. In human and nonhuman primates, MPTP produces an irreversible

and severe parkinsonian syndrome that replicates almost all the PD features including tremor, rigidity, slowness of movement, postural instability, and even freezing. The responses to traditional antiparkinsonian therapies are virtually identical to those seen in PD, as are the complications of such therapies. However, in PD the neurodegenerative process is believed to occur over several years, whereas MPTP produces a clinical condition consistent with "end-stage PD" in a few days *(7)*. Except for a few cases *(8a)*, no human pathologic material has been available. Thus, the comparison between PD and the MPTP model is largely limited to nonhuman primates *(9)*.

According to neuropathologic data, MPTP administration causes damage to the dopaminergic pathways identical to that seen in PD *(10)*, with a resemblance that goes beyond the degeneration of SNpc dopaminergic neurons. Like PD, MPTP causes greater loss of dopaminergic neurons in the SNpc than in the ventral tegmental area *(11,12)* and greater degeneration of dopaminergic nerve terminals in the putamen than in the caudate nucleus *(13)*. On the other hand, two typical neuropathologic features of PD have, until now, been lacking in the MPTP model. First, except for the SNpc, the other pigmented nuclei such as the locus coeruleus have been spared, according to most published reports. Second, the eosinophilic intraneuronal inclusions, called Lewy bodies, so characteristic of PD have thus far not been convincingly observed in MPTP-induced parkinsonism *(8a,9)*. Also worth noting is the fact that postmortem brain samples from PD patients *(14)* show a selective defect in the same mitochondrial electron transport chain complex that is affected by MPTP *(15,16)*. Abnormalities in parameters of oxidative stress in postmortem PD brain tissue suggest that this disease is caused by an overproduction of free radicals *(17)*, the same highly reactive tissue-damaging species that are suspected of being involved in MPTP-induced dopaminergic toxicity in vivo *(18–20)*. However, despite this impressive resemblance between PD and the MPTP model, MPTP has never been recovered from postmortem brain samples or body fluids of PD patients. Altogether, these findings are consistent with the hypothesis that MPTP does not cause PD but is an excellent experimental model. Accordingly, it can be speculated that elucidating MPTP molecular mechanism(s) should lead to important insights into the pathogenesis and treatment of PD.

1.3.1. Metabolism of MPTP

The metabolism of MPTP is a complex, multistep process *(21)*. After its systemic administration, MPTP, which is highly lypophilic, rapidly crosses the blood-brain barrier. Once in the brain, the pro-toxin MPTP is metabolized to 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) by the enzyme monoamine oxidase B (MAO-B) within nondopaminergic cells, and then (probably by spontaneous oxidation) to 1-methyl-4-phenylpyridinium (MPP^+), the active

toxic compound. Thereafter, MPP^+ is released (by an unknown mechanism) in the extracellular space. Brain inflow of MPTP, together with its transformation into MPP^+, determines the amount of MPP^+ available to enter dopaminergic neurons. Extracellular MPP^+ is then taken up by plasma membrane dopamine transporters, for which it has high affinity *(22)*.

Alterations in many of these MPTP metabolic steps modify MPP^+ potency. For instance, blockade of MAO-B by pargyline and deprenyl *(23)* or of dopamine transporters by mazindol *(24)* prevents MPTP-induced dopaminergic toxicity. Striatal content of MPP^+ is linearly and positively correlated with the magnitude of dopaminergic damage *(25)*.

1.3.2. Mechanism of Action of MPTP

Once inside dopaminergic neurons, MPP^+ is concentrated by an active process within the mitochondria *(26)*, where it impairs mitochondrial respiration by inhibiting complex I of the electron transport chain *(27,28)* through its binding at or near the same site as the mitochondrial poison rotenone *(29,30)*. The inhibition of complex I impedes the flow of electrons along the mitochondrial electron transport chain, leading to a deficit in adenosine triphosphate (ATP) formation. It appears, however, that complex I activity should be reduced >70% to cause severe ATP depletion *(31)* and that, in contrast to in vitro paradigms, in vivo MPTP causes only a transient 20% reduction in mouse striatal and midbrain ATP levels *(32)*. These findings raise the question as to whether MPP^+-related ATP deficit can be the sole factor underlying MPTP-induced dopaminergic neuronal death. Another consequence of complex I inhibition by MPP^+ is an increased production of free radicals, especially of superoxide *(33–35)*. From the aforementioned findings, it may be speculated that the initiation of MPP^+'s deleterious cascade of events results from energy failure and oxidative stress, which individually may not be sufficient to kill cells, but in combination may well be lethal. A similar scenario of interplay among mitochondrial dysfunction, energy failure, and oxidative stress has been postulated for PD *(36)*.

The importance of MPP^+-related superoxide production in dopaminergic toxicity in vivo is demonstrated by the fact that transgenic mice with increased brain activity of copper/zinc superoxide dismutase (SOD1) are significantly more resistant to MPTP-induced dopaminergic toxicity than their wild-type littermates *(18)*. This finding strongly suggests that the superoxide radical plays a pivotal role in the MPTP neurotoxic process. However, superoxide is poorly reactive, and it is the general consensus that this radical does not cause serious direct injury *(37)*. Instead, superoxide is believed to exert many or most of its toxic effects through the generation of other reactive species such as the hydroxyl radical, whose oxidative properties can ultimately kill cells *(37)*. For instance, superoxide facilitates hydroxyl radical production by hydrogen per-

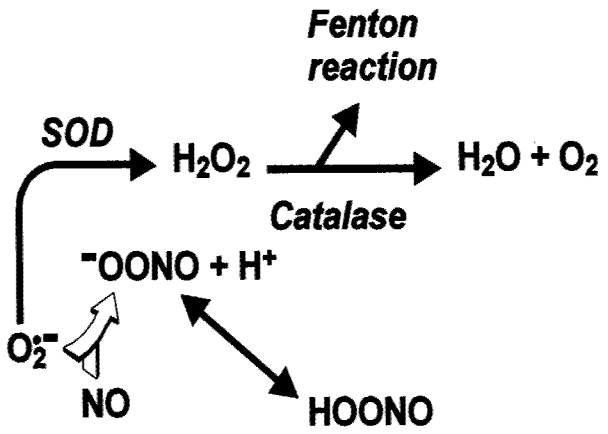

Fig. 1. Superoxide ($O_2^{\bullet-}$) can react with superoxide dismutase (SOD) to produce hydrogen peroxide (H_2O_2), which in turn can react with catalase to produce water and oxygen or enter the Fenton reaction. Alternatively, superoxide can also react with nitric oxide (NO) to produce peroxynitrite ($^-$OONO) and peroxynitrous acid (HOONO).

oxide and transitional metals such as iron (i.e., the Fenton reaction; **Fig. 1**) *(37)*. Although this reaction can readily take place in vitro, its occurrence in vivo is subordinate to such factors as low pH *(38)*. Despite this unfavorable pH constraint, MPTP does stimulate the formation of hydroxyl radicals *in vivo*, as evidenced by the increase in the hydroxyl radical-dependent conversion of salicylate into 2,3- and 2,5-dihydroxy-benzoates *(19)*.

Superoxide can also react with NO to produce peroxynitrite, another potent oxidant *(39)* (**Fig. 1**). At physiologic pH and in aqueous milieu, this reaction proceeds five times faster than the decomposition of superoxide by SOD *(40)* (see thick white arrow in **Fig. 1**). The intracellular concentration of SOD1 is estimated to be 10–40 μ*M* *(41)*. Thus, NO concentration has to be approximately 10 μ*M* for peroxynitrite formation to be competitive, which is not unrealistic as NO production at the cellular level is estimated at 1–10 μ*M* *(39)*. The situation is different, however, for superoxide, whose basal intracellular concentration is low *(42)*. Thus, under normal conditions, superoxide is limiting, and it is likely that minimal peroxynitrite formation occurs. Conversely, in pathologic conditions, should superoxide concentrations increase, as in response to MPTP administration, formation of appreciable amounts of peroxynitrite is expected. In light of this and of our previous work on superoxide *(18)*, we *(43)* and others *(19,20)* have assessed the role of NO in the MPTP neurotoxic process. These studies show that inhibition of NOS attenuates, in a dose-dependent fashion, MPTP-induced striatal dopaminergic loss in mice *(19,43)*. We also demon-

strate that 7-nitroindazole (7-NI), a compound that inhibits NOS activity without significant cardiovascular effects in mice *(44)*, is profoundly neuroprotective against MPTP-induced SNpc dopaminergic neuronal death *(43)*. The protective effect of the NOS antagonist 7-NI against MPTP-induced striatal and SNpc dopaminergic damage was subsequently demonstrated in monkeys *(20)*.

1.4. Proposed Mechanism of MPTP Action

From the above findings, the following scheme can be proposed to explain both selectivity and dopaminergic toxicity (**Fig. 2**): MPTP is converted to MPP^+, which is transported into dopaminergic neurons via the dopamine transporter. MPP^+ inhibits enzymes in the mitochondrial electron transport chain, resulting in ATP deficit and increased "leakage" of superoxide from the respiratory chain. Superoxide cannot readily transverse cellular membranes and so remains in the cell and organelle in which it is produced. In contrast, NO is membrane permeable and diffuses into neighboring neurons. If the neighboring cell has elevated levels of superoxide, then there is an increased probability of superoxide reacting with NO to form peroxynitrite, which is highly reactive, damaging lipids, proteins, and DNA. In this scheme, it is the site of generation of superoxide that determines whether a cell will succumb to NO- and peroxynitrite-mediated deleterious effects. Since dopaminergic neurons selectively accumulate MPP^+, which in turn stimulates superoxide production, these neurons are selectively at risk.

1.4.1. Source of NO and NO Synthase

As summarized above, there is strong evidence that NO participates in the MPTP neurotoxic process. Because MPTP selectively kills dopaminergic neurons, it is expected that the deleterious cascade of events that underlie the neurodegeneration takes place inside dopaminergic neurons. As illustrated in **Fig. 2**, there are experimental arguments to indicate that superoxide concentration is indeed increased inside dopaminergic neurons by MPP^+. However, NO is produced by NOS, which thus far has not been identified inside dopaminergic neurons in rodents; although this needs to be confirmed, low levels of NOS might be present in dopaminergic neurons in humans *(45)*. In contrast to their lack of NOS, at least in rodents, dopaminergic structures are surrounded by NOS-containing fibers and cell bodies in the striatum, and, to a lesser extent, in the SNpc *(45,46)*. Because NO is uncharged and lypophilic *(47)*, it is able to travel away from its site of synthesis and inflict remote cellular damage without the need for any export mechanisms. It is suggested that NO, which is highly diffusible, can travel in random directions up to 150–300 μm during the 5–15 s that correspond to its estimated half-life in physiologic and aqueous conditions *(47)*. Although this modeling may depart from the actual in vivo

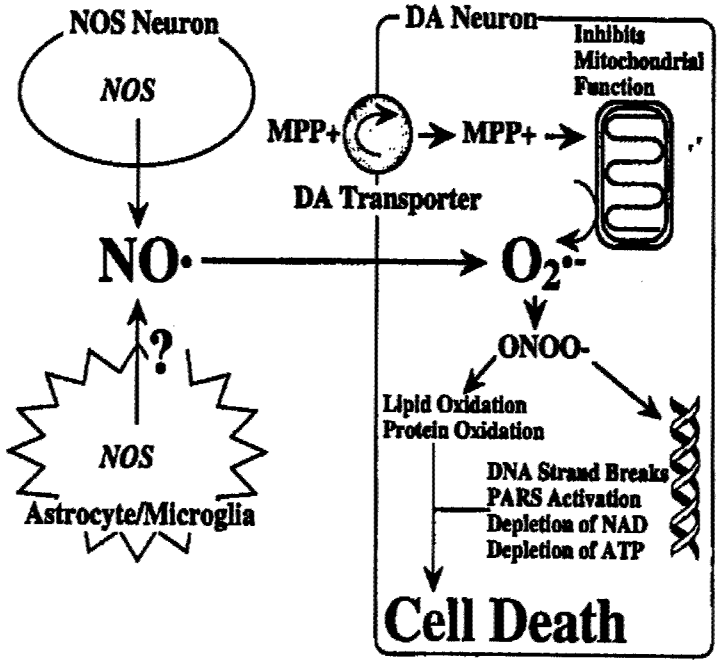

Fig. 2. Proposed scheme for selectivity of MPTP-induced dopaminergic neurotoxicity. DA, dopamine. (Reproduced with permission from **ref. *43***.)

situation encountered by a molecule of NO, it gives credence to the hypothesis that NO can cover a distance several times greater than the diameter of a cell. As shown in **Fig. 2**, we are thus speculating that the NO production involved in MPTP toxicity takes place in nondopaminergic cells present in the vicinity of dopaminergic structures.

Another question pertinent to the origin of NO in the MPTP model is which isoforms of NOS are primarily involved in this process? Nitric oxide is formed from arginine by NOS, which oxidizes the guanidino nitrogen of arginine, releasing NO and citrulline. To date, three distinct NOS isoenzymes have been purified and molecularly cloned (**Table 1**): neuronal NOS (nNOS, NOS I), inducible NOS (iNOS, NOS II), and endothelial NOS (eNOS, NOS III). These isoforms fall into two general categories on the basis of regulation of enzymatic activity. The first category comprises the constitutively expressed eNOS and nNOS isoforms, whose production of NO is Ca^{2+}/calmodulin dependent and is tightly controlled by mechanisms regulating intracellular Ca^{2+} levels. The second category comprises iNOS, which is not present constitutively (at least in most cells), but rather its mRNA transcription and translation are induced as part of an immune response in many cell types by endotoxin or

Table 1
Properties of NO Synthase Isoforms

First identified	Isoform	Name	Subcellular location	Denatured molecular mass	Regulation	Cofactors	No. amino acids	mRNA (kb)
Brain	I	Neuronal NOS (nNOS)	Soluble > particulate	160,000	Ca^{2+}/calmodulin	NADPH, FAD/FMN, tetrahydro-biopterin	1429 (rat)	10.5 (rat)
Macrophage	II	Inducible or macrophage NOS (iNOS)	Soluble > particulate	130,000	Inducible by cytokines, Ca^{2+} independent	Calmodulin, NADPH, FAD/FMN, tetrahydro-biopterin	1144 (mouse)	4.4–5.0 (mouse)
Endothelium	III	Endothelial NOS (eNOS)	Particulate > soluble	135,000	Ca^{2+}/calmodulin	NADPH, FAD/FMN, tetrahydro-biopterin	1203 (human)	4.3 (human)

inflammatory cytokines (e.g., γ-interferon, interleukin-1, or tumor necrosis factor). This inducible isoform contains calmodulin and is not regulated by intracellular changes in Ca^{2+} levels. Since all three isoforms of NOS have been identified in the brain, each of these can individually or in combination be involved in the production of NO used in MPTP neurotoxic process.

1.5. NOS Immunohistochemistry

There are at least two ways to explore the distribution of NOS proteins, by NOS immunohistochemistry or by nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase histochemistry; both approaches have advantages and disadvantages. However, rather than being exclusive, as we can see from the literature, both methods are often used within the same study.

1.5.1. Anti-NOS Antibodies

The main advantage of NOS immunohistochemistry over NADPH-diaphorase is that it allows the study of each NOS isoform separately. Anti-NOS antibodies directed against each NOS isoform are now commercially available; several of these, whether they are monoclonals or polyclonals, produce good results in immunohistochemistry as well as in Western blot.

The production of isoform-specific antibodies can be accomplished through a variety of approaches, including the production of monoclonal antibodies against purified protein, fusion protein, or synthesized peptides from the deduced amino acid sequence. Polyclonal antibodies directed against regions of the NOS isoforms with low homology can also be used to generate isoform-selective antibodies. Selection of peptides has been made easier by the development of computer programs that can assist in the design of peptides. Certain considerations are important to keep in mind. The peptide should be unique to your protein or at the very least have a low homology to the known isoforms. In addition, it should have a high antigenic index, and it is extremely helpful if it is water soluble. The methods, techniques, and theory behind the production of both monoclonal and polyclonal antibodies as well as the potential pitfalls that may arise in making antibodies are beyond the scope of this chapter and can be found in **ref. *48***.

1.5.2. Localization of NO Synthase Isoforms

Neuronal NOS is the predominant isoform of NOS in the brain. Its catalytic activity and protein are identifiable throughout the brain ***(45,49)***. Relevant to MPTP, nNOS is present in high density in the striatum within intrinsic medium-sized neurons colocalizing with somatostatin and neuropeptide Y ***(48)***. In the midbrain, nNOS is found in cholinergic neurons and within serotoninergic fibers ***(46,48)***. Thus, both by its abundance and its localization, nNOS appears

to be an excellent candidate for producing NO following MPTP. This is in agreement with our demonstration that mutant mice deficient in nNOS *(49)* are partially protected against MPTP-induced striatal dopaminergic toxicity *(43)*. The finding that mice are better protected by the NOS antagonist 7-NI than by the lack of nNOS expression suggests that nNOS is important but may not be the sole isoform of NOS involved in the MPTP neurotoxic process. Can it be iNOS?

In the normal brain, iNOS is not detectable *(50)* or is minimally expressed *(51)*. However, under pathologic conditions, iNOS expression can significantly increase in activated astrocytes as well as in other cells such as microglia *(52)*, invading macrophages, and even neurons *(53)*. This was shown in the brain after kainic acid lesion *(54)*, ischemic damage *(55)*, and stab wounds *(52)*. A similar scenario seems to exist in the MPTP model. Indeed, it appears that early in the course of MPTP-induced dopaminergic neuron degeneration there is an increase in striatal and midbrain iNOS activity (*55a*) consistent with the strong astrocytic and microglial reaction that occurs in these brain regions following MPTP administration *(55a,56,57)*. Changes in iNOS activity are already substantial 24 h after MPTP administration, which precedes the peak of dopaminergic neurodegeneration. Therefore, NO derived from iNOS may play a substantial role in MPTP-induced dopaminergic neurodegeneration. Since iNOS is induced following MPTP, it may not play a significant role in the initiation of the MPTP toxic process, but instead may amplify its propagation by fueling injured dopaminergic neurons with increasing amounts of NO. This view is consistent with our recent report of attenuated MPTP-induced SNpc degeneration in iNOS knockout mice (*55a*).

eNOS is localized in the endothelium of blood vessels in the periphery and in the nervous system. Aside from endothelial cells, eNOS has also been localized in different discrete regions of the brain by autoradiography *(58)* and immunostaining *(48)*. Although these studies report only low levels of eNOS in several brain regions, eNOS is particularly abundant in the hippocampus and in the olfactory bulb within neurons and neuropil. The presence of eNOS in the parenchyma, along with the fact that the brain is generously vascularized, makes it possible that eNOS contributes to the basal concentration of NO in the brain. As such, eNOS may have some importance in the MPTP neurotoxic process. Alternatively, its role in the vasculature *(59)* may be more significant for MPTP. As mentioned above, the striatal content of MPP^+, which is a determining factor in dopaminergic neurotoxicity, is tightly regulated by cerebral inflow of MPTP and outflow of MPP^+. Therefore, eNOS, by modulating blood vessel tone and thus cerebral perfusion, may alter the amount of blood-borne MPTP that gets into the brain and the kinetics of disappearance of MPP^+. Should the cause of PD be

related to an endogenous or exogenous blood-borne toxin, eNOS may be a therapeutic target for this disease.

1.6. NADPH-Diaphorase Histochemistry

Under appropriate conditions of paraformaldehyde fixation, NADPH-diaphorase staining can be used to identify all NOS isoforms. For unknown reasons, NOS is resistant to paraformaldehyde fixation, whereas all other NADPH-dependent oxidative enzymes are inactivated by fixatives.

1.6.1. Localization of NADPH-Diaphorase

In the brain, the highest density of NADPH-diaphorase is evident in the cerebellum and in the olfactory bulb ***(60,61)***. The accessory olfactory bulb has even more prominent staining. Other areas of high-density staining include the pedunculopontine tegmental nucleus, the superior and inferior colliculi, the supraoptic nucleus, the islands of Calleja, the caudate-putamen, and the dentate gyrus of the hippocampus. In the cerebellum, NADPH-diaphorase occurs in glutaminergic granule cells as well as in γ-aminobutyric acid (GABA)-ergic basket cells. In the cerebral cortex, NADPH-diaphorase staining colocalizes with somatostatin, neuropeptide Y, and GABA. In the corpus striatum, NADPH-diaphorase neurons also stain for somatostatin and neuropeptide Y. In the pedunculopontine tegmental nucleus of the brain stem, NADPH-diaphorase neurons stain for choline acetyltransferase but do not stain for somatostatin and neuropeptide Y. Even though there does not seem to be a single neurotransmitter that colocalizes with NADPH-diaphorase, all NADPH-diaphorase neurons identified co-localize with NOS ***(62)***. NOS catalytic activity accounts for diaphorase staining because transfection of cultured human kidney 293 cells with nNOS cDNA produces cells that stain for both nNOS and NADPH-diaphorase ***(62)***.

NADPH-diaphorase has been localized to the endothelium of blood vessels in the periphery and in the nervous system. NADPH-diaphorase is also concentrated in the hippocampus and is evident in pyramidal cells of the CA1 region and in granule cells of the dentate gyrus. eNOS or a closely related isoform accounts for NADPH-diaphorase staining in this structure. Using high concentrations of glutaraldehyde fixatives, we found that NADPH-diaphorase staining provides robust staining of pyramidal cells of the CA1 region.

NADPH-diaphorase has also been identified in neutrophils, macrophages, microglia, and astrocytes.

1.7. NOS Catalytic Activity

In several studies, it is important to gather information about the actual enzymatic activity of NOS. This question arises consistently when, for

example, one wishes to determine whether, in a pathologic condition such as PD, changes in NOS activity occur or how intense and lasting is the inhibition of NOS activity following administration of a NOS antagonist. Among the different methods available to assess NOS activity, the most reliable and straightforward is based on the conversion of [^{3}H]arginine into [^{3}H]citrulline (*see* **Subheading 3.**).

1.8. Peroxynitrite and Nitrotyrosine

Superoxide is produced by many biologic reactions, especially by mitochondrial respiration *(37)*. It can be engaged in numerous reactions including the direct oxidation of biological molecules (e.g., cathechols) and the production of hydroxyl radicals (**Fig. 1**). Similarly, NO exerts many biologic effects that can be defined as direct (i.e., resulting from the reactions between NO and specific biologic molecules) or indirect (i.e., resulting from the reactions between reactive nitric oxide species [RNOS], which are derived from NO oxidation, and specific biologic targets) *(41)*. Most, if not all, of the direct effects of NO appear to be related to biologic regulatory effects and not to neurotoxicity *(41)*; although NO can directly affect mitochondrial respiration in vitro *(63)*, the deleterious consequence of this effect remains to be determined in vivo. Conversely, the indirect actions of NO, which are mediated by RNOS such as nitrite (NO_2^-), nitrate (NO_3^-), and peroxynitrite and its protonated derivative peroxynitrous acid (N_2O_3H) are unquestionably deleterious *(41)*; in aqueous conditions, RNOS such as NO^+ and NO^- react rapidly with water and thus are unlikely to be major participants in noxious reactions.

In light of the above, it appears that since they are weak oxidants, neither superoxide nor NO is believed to be sufficiently damaging by themselves to participate directly in the MPTP toxic process. In contrast, peroxynitrite fulfils the role of the toxic mediator between superoxide and NO. The versatility of peroxynitrite as an oxidant is impressive: it can react with antioxidants (e.g., ascorbate, glutathione), unsaturated fatty acid in lipids, amino acid residues (e.g., cysteine, methionine, and tyrosine), and purine bases (e.g., guanine) *(64,65)*. By damaging DNA, peroxynitrite may stimulate the activity of poly(ADP-ribose) synthetase (PARP) *(66)*, which in turn may exacerbate MPP^+-induced ATP depletion *(66–68)*. One persistent fingerprint left by peroxynitrite is nitration of phenolic rings including tyrosine *(69)*. As such, detection and quantification of nitrotyrosine is important indirect evidence that peroxynitrite is involved in a pathologic process. Aside from being a marker, tyrosine nitration may also be deleterious, as it can inactivate enzymes and receptors that depend on tyrosine residues for their activity *(70,71)* and prevent phosphorylation of tyrosine residues important for signal transduction *(72,73)*.

This cascade of events appears quite relevant to the mode of action of MPTP, as we have demonstrated that, following MPTP administration to mice, both striatal and midbrain levels of nitrotyrosine in proteins increase in a time-dependent fashion and that tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, becomes inactivated by tyrosine nitration *(74)*. Nitrotyrosine has been successfully detected and quantified by different methods including high-performance liquid chromatography (HPLC), mass spectrometry, and immunobased assays *(75)*. Although these are not described in detail, we discuss them briefly and emphasize some important practical aspects.

1.8.1. Quantification of Nitrotyrosine

Several HPLC-based methods with ultraviolet (UV) or fluorescent detection have been used to quantify and separate chlorotyrosine and nitrotyrosine from tyrosine and other tyrosine analogs *(76,77)*. These techniques, however, like other UV or fluorescent detection-based methods, lack sensitivity. Two HPLC methods with electrodetection have recently been developed *(76,77)*; as anticipated, they are significantly more sensitive and, thus, more appropriate to quantify the minute amounts of nitrotyrosine presumably present in the brain. However, there are some concerns about whether these HPLC methods are actually detecting tyrosine analogs such as nitrotyrosine or an artifact *(78)*. Until this issue is resolved, it would be preferable to utilize a method that combines the possibility of quantifying tyrosine analogs such as isotope dilution gas chromatography combined with mass spectrometry (GC/MS) *(79–82)*. Relevant to the existence of peroxynitrite in the MPTP model, it has been demonstrated that MPTP significantly increases striatal levels of *free nitrotyrosine* in mice *(19)*. Although this finding provides major impetus to the implication of peroxynitrite in the MPTP model, one should be aware that the relationship between free and protein nitrotyrosine is unknown and that the physiopathologic role, if any, of free nitrotyrosine remains to be determined.

1.8.2. Visualization of Nitrotyrosine

Although the HPLC and GC/MS methods are highly sensitive, they do not provide information as to where at an anatomic or cellular level the changes in tyrosine nitration occur after MPTP administration. A similar issue was successfully addressed recently in spinal cord from amytrophic lateral sclerosis (ALS) patients *(83)* and in rejected transplanted kidney *(84)* in which levels of nitrotyrosine, determined by HPLC, were more than twofold higher in tissue extracts from these pathologic conditions compared with controls. In both situations, immunohistochemical analysis of nitrotyrosine revealed that conspicuous immunoreactivity was localized in spinal cord motor neurons *(83)* and in

kidney tubular cells *(84)*. Both locations are pertinent to the pathological processes that underlie ALS and chronic kidney transplant rejection. These immunohistochemical studies were performed by using specific antinitrotyrosine antibodies. Both mouse monoclonal and rabbit polyclonal antinitrotyrosine antibodies *(85)* are now commercially available from Upstate Biotechnology (UBI, Lake Placid, NY). Both were raised against nitrated keyhole limpet hemocyanin (KLH) and specifically recognize peroxynitrite-modified proteins including KLH, bovine serum albumin (BSA), catalase, histone, lysozyme, actin, rat brain homogenate, and heart homogenate, but not the corresponding native proteins. Nitrotyrosine (0.3 m*M*) can completely block the monoclonal or the polyclonal antibody binding to nitrated BSA; however, in routine use, concentrations as high as 10 m*M* nitrotyrosine may be necessary to block antibody binding fully. In contrast, a 10 m*M* concentration of either tyrosine, aminotyrosine, chlorotyrosine, hydroxytyrosine, or phosphotyrosine has no effect on antibody binding to nitrated BSA. It is, however, recommended to verify the specificity of the antibodies by two sets of controls on each tissue sample to confirm the presence of nitrotyrosine as follows: (1) blockade with an excess of nitrotyrosine; and (2) reduction to aminotyrosine with a strong reducing agent such as dithionite. Surprisingly, to our knowledge, to date no published studies exist regarding the identification of nitrotyrosine immunostaining in the MPTP model or in PD.

1.8.3. Target Proteins of Tyrosine Nitration in Dopamine Neurons

Aside from their use for immunostaining, the antinitrotyrosine antibodies have been used for immunoprecipitating and immunoblotting of tyrosine nitrated proteins *(74,86)*. These techniques are the cornerstone methods used to identify manganese SOD (SOD2) as a specific target of tyrosine nitration in the rejected kidney transplant *(84)*. This method also enabled us to demonstrate that TH is a major target of tyrosine nitration following MPTP administration *(74)*. Both immunoblot and the immunoprecipitation using the antinitrotyrosine antibodies are straightforward *(74)* and require the same type of controls as described for immunostaining.

2. Materials

Unless specified, all reagents required for the assays referenced in this chapter can be obtained from standard commercial sources and preferentially should be of the highest purity. Some technical comments and detailed recipes for the preparation of the necessary buffers and reaction solutions are given.

1. Water quality is a key factor to the success and reproducibility of the assays described in this chapter. Pure water refers to both low ion content and no infectious agents such as bacteria, fungi, and algae. This type of high-quality water

can be obtained from a distilled or a deionized water station. It is also advisable to autoclave distilled or deionized water whenever possible.

Caution: Some enzymatic reactions (e.g., horseradish peroxidase) may be inhibited by deionized water and thus, unless mandatory, it is advisable to use high-quality distilled water.

2. 0.2 *M* phosphate buffer (PB), pH 7.4**:** Prepare the following two stock solutions:
   a. Stock solution A: 0.2 *M* NaH_2PO_4 (12 g NaH_2PO_4 in 500 mL H_2O, or 13.9 g of $NaH_2PO_4 \cdot H_2O$ in 500 mL H_2O).
   b. Stock solution B: 0.2 *M* Na_2HPO_4 (28.4 g Na_2HPO_4 in 1 L H_2O).

   To make 0.2 *M* PB at pH 7.4: mix 1 part of solution A with 4 parts of solution B (e.g., 100 mL of A + 400 mL of B). This stock solution can be used to prepare the fixative solution (*see* **step 4**) or be diluted with an equal volume of distilled water to provide 0.1 *M* PB.
3. Tris-buffered saline (TBS): 50 m*M* Tris-HCl, pH 7.2 and 1.5% NaCl. Store at room temperature.
4. Fixative solution (4% paraformaldehyde/0.1 *M* PB): Depolymerize 8% paraformaldehyde in distilled H_2O (8 g in 100 mL H_2O) by heating to 80°C for 30 min while stirring. Do not exceed 80°C. If paraformaldehyde boils, start over.

   Caution: Wear gloves and mask while weighing and preparing paraformaldehyde. The solution must be heated in a fume hood.
   a. Clear paraformaldehyde solution with 1–2 drops of 10 *M* NaOH. *Solution will turn from a turbid appearance to a clear solution.*
   b. Let solution cool to room temperature (RT), check volume, and replace any H_2O that may have evaporated.
   c. Add an equal volume of 0.2 *M* PB, pH 7.4, to make a final solution of 4% paraformaldehyde/0.1 *M* PB.
   d. Filter 4% paraformaldehyde/0.1 *M* PB solution through a 0.22-μm filter unit and use within 1 wk (older 4% paraformaldehyde/0.1 *M* PB solution may contain crystals of formalin, which may cause tissue artifacts).
5. NADPH staining solution: Prepare buffer solution and stain mix as follows:
   a. Buffer solution: 0.1 *M* Tris-HCl, pH 7.2, 0.2% Triton X-100, 0.02% NaN_3.
   b. Stain Mix: 1 m*M* β-NADPH reduced form (8.33 mg in 10 mL buffer solution), 0.2 m*M* nitroblue tetrazolium (NBT) (1.64 mg in 10 mL buffer solution). Dissolve β-NADPH and NBT in the buffer solution in separate tubes. β-NADPH is readily soluble in the buffer, but NBT may need to be sonicated for 10 min. Mix β-NADPH and NBT together in a 1:1 ratio and filter before application to cells. The final concentration of the stain mix should be 1 m*M* β-NADPH reduced form and 0.2 m*M* NBT. Since the two compounds are solubilized in separate tubes, each tube should contain a 2X concentration, so that when mixed together in a 1:1 ratio the final concentration is 1X. *The final staining mix should be filtered before use to prevent formation of dark blue precipitates.*
6. NOS enzymatic activity reaction mix: Prepare buffer solution and reaction mix as follows:
   a. Reaction buffer: 50 m*M* Tris-HCl, pH 7.4, 2.5 m*M* dithiothreitol (DTT; 3.8 mg/mL), 10 μ*M* tetrahydrobiopterin (BH_4; 3.1 mg/mL). *Although Tris-*

HCl can be kept at 4°C for an extended period, DTT and BH_4 must be added to Tris-HCl on the day of the assay.

b. NADPH solution (1.25 m*M*): Dissolve 5 mg of NADPH in 5 mL of reaction buffer. This tube must be protected from light and kept on ice.
c. $CaCl_2$ solution (28 m*M*): Dissolve 78 mg of $CaCl_2$ in 5 mL reaction buffer.
d. Radioactive solution: Mix 12.5 µL L-[2,3-^{3}H]arginine (specific activity: 36.8 Ci/mmol; Dupont-NEN, Boston, MA) with 1250 µL reaction buffer. This tube must be protected from light and kept on ice. *Ten microliters of this radioactive mix should produce 250,000–500,000 dpm.*
e. Stopping buffer: 20 m*M* HEPES, pH 5.5, 1 m*M* EDTA, and 1 m*M* EGTA.
f. Resin suspension: Wash 100 g Dowex AG50WX-8 (Pharmacia, Piscataway, NJ) resin (hydrogen form) with 200 mL 1 *N* NaOH to convert it from the hydrogen to the Na form. Gently stir the mixture for 15 min at RT, let the mix settle, then pour off the liquid, and repeat the NaOH wash two more times. Afterward, wash the resin several times with distilled water until the pH (use pH paper) of the resin suspension is between 7.0 and 8.0. Then decant and resuspend the resin with stopping buffer. *The prepared resin suspension can be kept for several weeks in a beaker covered with parafilm at 4°C.*

3. Methods

3.1. NOS Immunostaining Protocol

1. Anesthetize rats or mice with pentobarbital (35–45 mg/kg intraperitoneal injection) and perfuse them with cold saline followed by 4% paraformaldehyde/0.1 *M* PB containing 0.1% glutaraldehyde (250 mL for rats and 75 mL for mice).
2. Remove the brains and postfix tissues for 2 h in 4% paraformaldehyde/0.1 *M* PB.
3. Cryoprotect brains by immersing them in 20% (v/v) glycerol in PB. Alternatively, the brains can be cryoprotected in 30% (v/v) sucrose in PB by sequentially bathing the tissue in 10, 20, and 30% sucrose.
4. Permeabilize slide-mounted or free-floating tissue sections in TBS containing 0.4% Triton X-100 for 30 min at RT (*see* **Note 1**).
5. Block tissue sections for 1 h in TBS containing 4% normal goat serum (NGS), 0.2% Triton X-100, and 0.02% NaN_3 at RT.
6. Incubate sections overnight at 4°C in TBS containing 0.1% Triton X-100, 2% NGS, and 0.02% NaN_3 with the appropriate dilution of primary antibody.
7. Rinse tissue sections with TBS containing 1% NGS (3 X 10 min).
8. Incubate tissue sections with a biotinylated goat-anti-rabbit antibody (Vector) at a 1:200 dilution for 1 h at RT in TBS containing 1.5% NGS.
9. Rinse tissue sections 2 X 10 min in TBS containing 1% NGS and then 2 X 10 min in TBS.
10. Incubate tissue sections with an avidin-biotin-horseradish peroxidase complex (Vector Elite, Vector) at a 1:50 dilution in TBS for 1 h at RT.
11. Rinse tissue sections 3 X 10 min in TBS.
12. Develop color with a substrate solution consisting of 0.1% H_2O_2 and 0.5 mg/mL diaminobenzidine in TBS.
13. Rinse section with TBS.

14. Mount sections on gelatin-coated glass slides and allow to air dry prior to counterstaining (facultative) with thionin, which gives a light blue/purple color to the nucleus of all cells and to Nissl bodies of neurons.
15. Dehydrate the sections and coverslip using Permount (Fisher) (*see* **Note 2**).

3.2. NADPH-Diaphorase Histochemistry

1. Anesthetize rats or mice with pentobarbital, perfuse, and process brains as described for NOS immunohistochemistry (*see* **Subheading 3.1., steps 2–4**).
2. Incubate sections at 37°C in the NADPH-diaphorase staining mix.
3. Check staining at 30 min under a light microscope.
4. Let stain for 30–90 min. Cells that contain NOS should turn purple/dark blue.
5. Stop the reaction by rinsing the tissue sections (3 X 10 min) in cold TBS (*see* **Note 3**).
6. Mount sections on gelatin-coated glass slides.
7. If counterstaining is required, use any dye that produces a color other than blue/purple such as crystal green.
8. Dehydrate sections and coverslip using Permount (Fisher) (*see* **Note 2**).

3.3. NOS Catalytic Activity

3.3.1. Tissue Preparation

1. Sacrifice rats or mice by decapitation and quickly remove the brains.
2. Dissect out regions of interest such as striatum and cerebellum freehand on an ice-cold glass Petri dish *(87)*.
3. Immediately freeze the samples on dry ice and store at –80°C until analysed.

3.3.2. Radioenzymatic Assay

nNOS and eNOS catalytic activity are assayed by measuring Ca^{2+}-dependent activity; iNOS is assayed by measuring the Ca^{2+}-independent conversion of [^{3}H]arginine to [^{3}H]citrulline *(49)*.

1. On the day of the assay, sonicate frozen tissue samples in 20 volumes (w/v) 50 m*M* Tris-HCl, pH 7.4, buffer containing 1 m*M* EDTA and 1 m*M* EGTA and keep on ice.
2. Add 25 µL of homogenate to the various reaction tubes, which should be placed on ice and prepared as described in **Table 2** (*see* **Note 4**).
3. Incubate tubes for 15 min at 25°C.
4. Terminate the reaction by the addition of 3 ml of cold stopping buffer.
5. Apply the total resulting volume progressively to a mini-column packed with 0.5–1.0 mL Dowex AG50WX-8 resin.
6. Quantify [^{3}H]citrulline by liquid scintillation counting of the eluate. For each experiment, the blank (background) is generated by omitting NADPH (*see* **Note 2**).

4. Notes

1. Although we use TBS, phosphate-buffered saline would give the same result in most instances. However, be aware that many enzymes, including horseradish

Table 2
Experimental Tubes (Duplicate) for NOS Activity Assay[a]

	Ca^{2+}-dependent activity		Ca^{2+}-independent activity		Blank	
	1	2	1	2	1	2
Tris buffer	0	0	10	10	90	90
Radioactive	10	10	10	10	10	10
$CaCl_2$	10	10	0	0	0	0
Homogenate	25	25	25	25	25	25
NADPH	80	80	80	80	0	0
Total volume	125	125	125	125	125	125

[a]All volumes are in µL. NADPH starts the reaction, so it should be added last.

peroxidase, can be inhibited by phosphate. Thus, unless required, TBS would be the preferred buffer.

2. In case of failure in any of the assays described above, do not dismiss the possibility that the culprit could be the water (e.g., inhibition caused by deionized water, infection), buffer (e.g., incorrect concentration or pH), and degradation or omission of the primary or secondary antibodies as well as key reagents such as NADPH.
3. For NADPH-diaphorase, be aware that when the solution turns pink/purple, the reaction should be stopped. Optimal NADPH-diaphorase staining is usually obtained prior to the solution turning pink/purple.
4. For NOS activity, if only nNOS activity is to be studied, then it is preferable to centrifuge the tissue homogenate (18,000*g*, 15 min, 4°C) and use 25 µL of the supernatant for the reaction since nNOS is a soluble enzyme.

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REVIEW ARTICLE

The parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): a technical review of its utility and safety

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Parkinson's disease (PD) is a common disabling neurodegenerative disorder the cardinal clinical features of which include tremor, rigidity and slowness of movement (Fahn and Przedborski 2000). These symptoms are attributed mainly to a profound reduction of dopamine in the striatum due to a dramatic loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Fahn and Przedborski 2000). Thus far, both the cause and the mechanisms of PD remain unknown. Over the years, investigators have used experimental models of PD produced by several compounds such as reserpine, 6-hydroxydopamine, methamphetamine, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to provide insights into the mechanisms responsible for the demise of dopaminergic neurons in PD. To this end, MPTP has emerged unquestionably as a popular tool for inducing a model of PD in a variety of animal species including monkeys, rodents, cats, and pigs (Kopin and Markey 1988). The sensitivity to MPTP and therefore its ability to induce parkinsonism closely follows the phylogenetic tree where the species most closely related to humans are the most vulnerable to this neurotoxin. Due to the significant neurotoxicity of MPTP, it is important that researchers appreciate the potential hazards of this toxin. Given this, the purpose of this review is to inform the researcher of the hazardous nature of MPTP and to provide guidance for its safe handling and use.

MPTP models of PD

MPTP is a by-product of the chemical synthesis of a meperidine analog with potent heroin-like effects. MPTP can induce a parkinsonian syndrome in humans almost indistinguishable from PD (Langston and Irwin 1986). Recognition of MPTP as a neurotoxin occured early in 1982, when several young drug addicts mysteriously developed a profound parkinsonian syndrome after the intravenous use of street preparations of meperidine analogs which, unknown to anyone, were contaminated with MPTP (Langston *et al.* 1983). In humans and non-human primates, depending on the regimen used, MPTP can produce an irreversible and severe parkinsonian syndrome that replicates almost all of the features of PD, including tremor, rigidity, slowness of movement, postural instability, and even freezing; in non-human primates, a resting tremor characteristic of PD has only been demonstrated convincingly in the African green monkey (Tetrud *et al.* 1986). The responses, as well as the complications, to traditional antiparkinsonian therapies are virtually identical to those seen in PD. It is believed that in PD the neurodegenerative process occurs over several years, while the most active phase of neurodegeneration is completed within a few days following MPTP administration (Langston 1987;

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Abbreviations used: MAO-B, monoamine oxidase B; $MPDP^+$, 1-methyl-4-phenyl-2,3-dihydropyridinium; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MSDS, material safety data sheet, PD, Parkinson's disease; PPE, personal protective equipment; SNpc, substantia nigra pars compacta.

Jackson-Lewis *et al.* 1995). However, recent data suggest that, following the main phase of neuronal death, MPTP-induced neurodegeneration may continue to progress 'silently' over several decades, at least in humans intoxicated with MPTP (Vingerhoets *et al.* 1994; Langston *et al.* 1999). Except for four cases (Davis *et al.* 1979; Langston *et al.* 1999), no human pathological material has been available for studies and thus, the comparison between PD and the MPTP model is largely limited to primates (Forno *et al.* 1993). Neuropathological data show that MPTP administration causes damage to the nigrostriatal dopaminergic pathway identical to that seen in PD (Agid *et al.* 1987), yet there is a resemblance that goes beyond the loss of SNpc dopaminergic neurons. Like PD, MPTP causes greater loss of dopaminergic neurons in SNpc than in ventral tegmental area (Seniuk *et al.* 1990; Muthane *et al.* 1994) and, at least in monkeys treated with low doses of MPTP but not in humans, greater degeneration of dopaminergic nerve terminals in the putamen than in the caudate nucleus (Moratalla *et al.* 1992; Snow *et al.* 2000). However, two typical neuropathologic features of PD have, until now, been lacking in the MPTP model. First, except for SNpc, pigmented nuclei such as locus coeruleus have been spared, according to most published reports. Second, the eosinophilic intraneuronal inclusions, called Lewy bodies, so characteristic of PD, thus far, have not been convincingly observed in MPTP-induced parkinsonism (Forno *et al.* 1993), although, in MPTP-injected monkeys, intraneuronal inclusions reminiscent of Lewy bodies have been described (Forno *et al.* 1986).

Modes of administration

To date, the most frequently used animals for MPTP studies are monkeys, mice and rats. The administration of MPTP through a number of different routes using different dosing regimens has led to the development of several distinct models, each characterized by some unique behavioral and/or biochemical features. The manner in which these models were developed is based on the concept of delivering MPTP in a fashion that creates the most severe and stable form of SNpc damage with the least number of undesirable consequences such as acute death, dehydration and malnutrition. Although MPTP can be given by a number of different routes, including gavage and stereotaxic injection into the brain, the most common, reliable, and reproducible lesion is provided by its systemic administration (i.e. subcutaneous, intravenous, intraperitoneal or intramuscular).

Monkeys

The most commonly used regimens in monkeys are the multiple intraperitoneal or intramuscular injections and the intracarotid infusion of MPTP (Petzinger and Langston 1998). The former is easy to perform and produces a bilateral parkinsonian syndrome. However, often the monkey exhibits a generalized parkinsonian syndrome so severe that chronic administration of levodopa is required to enable the animal to eat and drink adequately (Petzinger and Langston 1998). On the other hand, the unilateral intracarotid infusion is technically more difficult, but causes symptoms mainly on one side (Bankiewicz *et al.* 1986; Przedborski *et al.* 1991), which enables the monkey to maintain normal nutrition and hydration without the use levodopa.

For many years monkeys were mainly, if not exclusively, treated with harsh regimens of MPTP to produce an acute and severe dopaminergic neurodegeneration (Petzinger and Langston 1998). More recently, several investigators have treated monkeys with low doses of MPTP (e.g. 0.05 mg/kg 2–3-times per week) for a prolonged period of time (i.e. weeks to months) in an attempt to better model the slow neurodegenerative process of PD (Schneider and Roeltgen 1993; Bezard *et al.* 1997; Schneider *et al.* 1999). While both the acute and the chronic MPTP-monkey models are appropriate for the testing of experimental therapies aimed at alleviating PD symptoms, it is the chronic model that is, presumably, the most suitable for the testing of neuroprotective strategies.

Mice

In addition to monkeys, many other mammalian species are also susceptible to MPTP (Kopin and Markey 1988; Heikkila *et al.* 1989; Przedborski *et al.* 2000). Mice have become the most commonly used species for both technical and financial reasons. However, several problems need to be emphasized. First, mice are much less sensitive to MPTP than monkeys; thus, much higher doses are required to produce significant SNpc damage in this animal species, presenting a far greater hazardous situation. Second, in contrast to the situation in monkeys, mice treated with MPTP do not develop parkinsonism. Third, the magnitude of nigrostriatal damage depends on the dose and dosing schedule (Sonsalla and Heikkila 1986).

Rats

The use of MPTP in rats presents an interesting situation (Kopin and Markey 1988). For instance, rats injected with mg/kg doses of MPTP comparable to those used in mice do not exhibit any significant dopaminergic neurodegeneration (Giovanni *et al.* 1994a; Giovanni *et al.* 1994b). Conversely, rats injected with much higher doses of MPTP do exhibit significant dopaminergic neurodegeneration (Giovanni *et al.* 1994a; Giovanni *et al.* 1994b) although, at these high doses, rats have to be pretreated with guanethidine to prevent dramatic peripheral catecholamine release and extensive mortality (Giovanni *et al.* 1994a). These findings indicate that rats are relatively insensitive to MPTP, but regardless of this drawback, rats continue to be used often in MPTP studies (Storey *et al.* 1992; Giovanni *et al.* 1994a; Giovanni

et al. 1994b; Staal and Sonsalla 2000; Staal *et al.* 2000). In rats, the systemic administration of MPTP is rarely used and the vast majority of studies involve the stereotaxic infusion of MPTP's toxic metabolite, 1-methyl-4-phenylpyridinium (MPP^+) (Storey *et al.* 1992; Giovanni *et al.* 1994a; Giovanni *et al.* 1994b; Staal and Sonsalla 2000; Staal *et al.* 2000).

Intervening factors

Several factors influence the reproducibility of the lesion in monkeys, rats, and mice. However, to our knowledge, the extensive and systematic assessment of these factors has only been done in mice, and can be found in the following references (Heikkila *et al.* 1989; Giovanni *et al.* 1991; Giovanni *et al.* 1994a; Giovanni *et al.* 1994b; Miller *et al.* 1998; Hamre *et al.* 1999; Staal and Sonsalla 2000), the highlights of which can be summarized as follows: different strains of mice (and even within a given strain obtained from different vendors) can exhibit strikingly distinct sensitivity to MPTP. This differential sensitivity acts in an autosomal dominant fashion (Hamre *et al.* 1999). Gender, age, and body weight are also factors that modulate MPTP sensitivity as well as reproducibility of the lesion, in that female mice are less sensitive and exhibit more variability in the extent of damage than males, as do mice younger than 8 weeks and lighter than 25 g. From our experience, optimal reproducibility in MPTP neurotoxicity is obtained using male C57 BL/6 mice 8–10 weeks of age and 25–30 g in weight. Also of importance is that, following MPTP administration, some mice will die within the first 48 h postinjection; note that C57 BL/6 mice from different vendors exhibit dramatically different magnitudes of acute lethality, ranging from 5% to 90%. This common issue is unlikely related to a toxic effect in the central nervous system but rather toxicity to the peripheral nervous and cardiovascular systems. Although, to our knowledge, this possibility has never been formally studied, we believe that, following acute MPTP administration, mice develop fatal alterations in heart rate and blood pressure. Moreover, MPTP intoxication causes a transient drop in body temperature, which not only can modulate the extent of dopaminergic damage (Moy *et al.* 1998), but can also contribute to acute lethality. Death rate can be reduced by maintaining the body temperature of the injected mice using a temperature-controlled warming pad (do not use a lamp, which can kill mice by overheating them as there is no control of the temperature).

Metabolism of MPTP

MPTP has a complex multistep metabolism (Tipton and Singer 1993; Przedborski *et al.* 2000). It is highly lipophilic, and freely and rapidly crosses the blood–brain barrier. Within a minute after MPTP injection, levels of the toxin are detectable in the brain (Markey *et al.* 1984). Once in the brain, MPTP is metabolized to 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) by the enzyme monoamine oxidase B (MAO-B) in non-dopaminergic cells. Then $MPDP^+$ is oxidized to the active MPTP metabolite, MPP^+, which is then released into the extracellular space, where it is taken up by the dopamine transporter and is concentrated within dopaminergic neurons, where it exerts its toxic effects. The essential role of these different metabolic steps in MPTP-induced neurotoxicity and the fact that MPP^+ is the actual culprit are demonstrated by the following observations: (1) pretreatment with MAO-B inhibitors such as deprenyl prevents MPTP biotransformation to MPP^+ and blocks dopaminergic toxicity (Heikkila *et al.* 1984; Markey *et al.* 1984); (2) pretreatment with dopamine uptake inhibitors (e.g. mazindol) prevents MPP^+ entry into dopaminergic neurons and also blocks dopaminergic toxicity (Javitch *et al.* 1985), at least in mice; and (3) striatal MPP^+ content correlates linearly with dopaminergic toxicity in mice (Giovanni *et al.* 1991).

Body distribution and environmental contamination

Knowing where MPTP and its toxic metabolite, MPP^+, accumulate both inside and outside of the body of the injected animal following MPTP administration is germane to the formulation of any set of standard practices for the safe use of MPTP.

Following MPTP administration to both mice and monkeys, only the interior surfaces of the cage, the surfaces that the animals and/or their excreta could physically touch, including food and drinking bottle, are contaminated with MPTP and its metabolites (Yang *et al.* 1988). Conversely, no evidence of contamination is found outside of the cage or on the outside surrounding surfaces (Yang *et al.* 1988). At two days postinjection, 70% of the total injected dose of MPTP and its metabolites is recovered from the inside cage-wash, urine and feces, of which about 15% in mice and 2% in monkeys is unmetabolized MPTP, while the rest is due to MPTP metabolites, such as MPP^+. Moreover, it appears that the excretion of unmetabolized MPTP occurs mainly during the first day postinjection, while mainly MPTP metabolites are excreted up to 3 days postinjection (Yang *et al.* 1988). There is no evidence either in mice or in monkeys that MPTP and its metabolites are still being excreted after 3 days post MPTP administration. Although high concentrations of MPTP are found in the bile, the main route of MPTP excretion is the urine (Johannessen *et al.* 1986). MPTP in urine will likely be ionized and not volatile, and be well absorbed by the animal bedding. Also, less than 0.01% of the total injected dose of MPTP is detected as volatile MPTP, which probably originates from the animals exhaling MPTP or from vapors from contaminated urine (Yang *et al.* 1988).

One day after an injection of radiolabeled MPTP to mice, most of the radioactivity is localized in the brain and the adrenal gland, while all other organs contain 50–75% lower amounts of radioactivity (Johannessen *et al.* 1986). Analysis of the radioactive species recovered from different organs and body fluids such as bile, urine, blood, and CSF demonstrates variable amounts of unmetabolized MPTP soon after injection, but by 12–24 h postinjection, essentially all of the radioactivity corresponds to MPP^+ (Markey *et al.* 1984; Johannessen *et al.* 1986).

From the above, it appears that the potential risks of exposure to MPTP are through direct contact with the animal, the animal cage inner surfaces, and its bedding material. There is minimal risk from exposure due to airborne or vapor-borne forms of MPTP. Although safety procedures, as outlined below, must always be followed, the period of maximal risk of MPTP contamination is from the moment of injection to the time that MPTP or its metabolites are no longer found in the excreta of treated animals; as a precautionary measure, we recommend extending the period of high-risk from 3 days to 5 days post-MPTP injection.

Personal protection

Prior to discussing MPTP preparation, injection and animal experimentation, it is necessary to discuss the issues of the recommended facility and personal protective equipment (PPE). As a rule, only investigators and/or staff members who are trained in handling hazardous agents and who are familiar with MPTP safety procedures and practices should prepare and administer MPTP, and monitor the animals during the high-risk period (i.e. 5 days post-MPTP injection). Of note, any staff member who undertakes these tasks should give informed consent and not be coerced into taking on MPTP-related duties. Moreover, it is strongly recommended that all aspects of the MPTP experiment, including storage and solution preparation, take place in a dedicated procedure room (for small animals) or area within the animal room (for large animals), and not in a regular laboratory. For personal safety, when using MPTP, researchers are required to wear the PPE described below, during the preparation of the MPTP solution, the injection period, and 5 days postinjection. Thereafter, regular laboratory attire as required to handle animals is sufficient.

It is important to emphasize that in laboratories committed to MPTP research, one cannot exclude that exposure to even trace amounts of MPTP over many years of the same investigator and/or staff member may have negative consequences. This is one more reason why a heightened standard of protection must be implemented for any individual involved in MPTP experiments.

Dedicated procedure room and area within animal room

All MPTP experiments including preparation of solutions must be performed in a procedure or animal room under negative-pressure because aerosols from MPTP and its metabolites can be generated from bedding, excreta and animal fur. All animals should be acclimated to the room for 4–7 days prior to any MPTP experiment to allow for monoamine stabilization before MPTP injection since monoamine level alterations may affect intragroup lesion reproducibility. The procedure or animal room should have a 12-h light-dark cycle, a bench with a working area, a sink, and be temperature-controlled. For small animals like mice, it should also be equipped with an animal rack to hold all of the cages and a fume hood. All furniture should be of stainless steel or of any material, except wood, that is acid-resistant and washable. All working surfaces including the fume hood and animal racks should be covered with materials that are absorbent on the face-up side and non-absorbent on the face-down side. The entire floor of the procedure room or working area in the animal room for large animals should be covered with plastic-backed absorbent sheets. A warning sign clearly stating 'Danger! MPTP Neurotoxin Use Area – Entry Restricted' must be posted on the outside of the procedure or animal room door. The room must be locked at all times and the animal care staff informed of the ongoing use of MPTP and its dangers. They must also be informed that this room is off limits unless allowed to enter by the responsible investigator.

This procedure room or designated procedure area should be completely equipped with all of the necessary supplies for the MPTP experiments. It should also contain a sharps disposal container clearly labeled as hazardous waste, a container lined with a hazardous waste disposal bag for solid waste (diapers, gloves, animal shavings, etc.), gloves, absorbent pads, paper towels, markers, weighing scales for animals and MPTP, sterile saline, syringes with needles, 1% bleach (sodium hypochlorite) solution in water, a strong biodegradable detergent, personal protective equipment (see below), and deprenyl (selegiline), an MAO-B inhibitor, for accidental exposure to MPTP. It is imperative that the material safety data sheet (MSDS) for MPTP, which is supplied by the manufacturer, be kept in the room. Thus, once in the room or area, there should be no need to exit during the injection period.

Personal protection equipment

PPE must be worn during all procedures involving MPTP, including during the 5 days post-MPTP injection. The PPE is far more important when injecting mice than monkeys as mice require significantly higher doses of MPTP. The PPE consists of a one-piece garment with an

attached hood, elasticized wrists and attached boots made of a lightweight, chemically and biologically inert, non-absorbent material that is tear-resistant and provides protection from airborne particles. This garment should be easy enough to get into and economical enough to throw away after one wearing. For example, coveralls made of Tyvek fabric with elasticized wrists and boots and an attached hood (Kapplar, Guntersville, AL, USA) can be used. A full-face respirator with removable HEPA filter cartridges that is fit-tested to the individual is preferred for facial and respiratory protection. Alternatively, a half-face air-purifying respirator with removable HEPA cartridges that is approved by the National Institute of Occupational Safety and Health (NIOSH)/Mine Safety Health Administration (MSHA) for respiratory protection against dusts that is fit-tested to the individual using the respirator can also be used. The respirator is re-usable and should be thoroughly wiped with 1% bleach solution then washed with detergent after each use; wipes must be disposed of in the hazardous waste container. Splash-proof goggles and double-layered nitrile under latex gloves complete the PPE attire. All items comprising the PPE attire can be obtained from a large general laboratory supply company. The office of environmental health and safety in any Institution where MPTP is to be used must be consulted for guidance in obtaining PPE attire for use with MPTP.

Housing

For small animals such as mice, disposable cages and accessories are strongly recommended as they permit incineration of waste without bedding changes. Covering cages with filter bonnets is recommended to significantly reduce both room contamination and cross contamination of other animals. Small animal cages should be placed on the animal rack in the procedure room prior to and during the five-day period post-MPTP injection. All injections must be performed in the fume hood in the procedure room.

For large animals such as monkeys, enclosed cages should be used. The base of the cage and the drop pan must be lined with plastic-backed absorbent pads.

MPTP storage and handling

MPTP can be purchased from several commercial sources. Unless specifically required, do not use MPTP as the free base, but only as the hydrochloride or other non-volatile salt conjugate. MPTP storage and handling must be restricted to the procedure room or designated area within the animal room. Minimize the use of large volumes, concentrated solutions, and handling of MPTP powder and never transport MPTP solutions or opened vials of MPTP outside of the dedicated room. MPTP may be purchased in small quantities of 10 mg or 100 mg in glass septum bottles. Vials of MPTP must be kept closed until used and stored at room temperature in a container within a vacuum-sealed desiccated container. This second container should be kept in a locked cabinet with a permanently affixed 'MPTP – Neurotoxin' label. This cabinet must be secured to a non-removable surface in the procedure room or area.

Only investigators appropriately trained in the handling of MPTP should perform manipulations involving the powder. Use of glass containers will reduce handling problems that result from the electrostatic properties of plastic. It is strongly recommended that a balance dedicated to weigh MPTP powder be kept in the procedure room. Prior to weighing MPTP powder, cover the weighing area with pads dampened with 1% bleach solution to reduce the risk of airborne MPTP powder particles. To minimize the risk of MPTP powder spills, the weighing procedure described by Pitts *et al.* (1986) is a safe method: tare a small container (e.g. small scintillation glass vial with a screw cap); take the tared container and place an approximated amount of MPTP in it, close and wipe container with 1% bleach solution; weigh container; then add solvent to give desired concentration; again wipe container and all other items with 1% bleach solution; dispose of all wastes in a hazardous waste container. Alternatively, if a given experiment requires a total daily dose of less than 10 mg or 100 mg of MPTP, then it is safer not to open the vial and weigh the powder but to add the desired volume of solvent/vehicle directly to the sealed 10 mg or 100 mg vial. It must be understood that this MPTP solution has to be used in one day and the remainder discarded since MPTP in solution oxidizes at room temperature; prior to discarding the used MPTP sealed vial, inject a volume of 1% bleach solution equivalent to the volume of MPTP solution remaining into the vial, then discard the vial as biohazardous liquid waste. We previously found that storing MPTP solution at −80°C retards its oxidation as MPTP solution appears stable up to 2 months (personal observation). However, unless one has a dedicated −80°C freezer for MPTP storage, other issues such as laboratory safety will arise and that even without mentioning the negative impact of thawing and freezing of MPTP solution on its neurotoxic potency.

Animals should be injected only with sterile solutions of MPTP prepared by either filtration through a disposable 0.22 μm filter unit or by dissolving the compound in sterile saline or water. Do not autoclave MPTP solutions, as this will vaporize the compound and may lead to exposure from inhalation.

Injection of MPTP

As mentioned above, a number of different injection regimens have been used to produce the desired MPTP lesions. These are based on a number of factors, including experimental design, degree of desired lesion, and species

used. As indicated, mice, which typically require greater amounts of MPTP to produce lesions, can be injected either subcutaneously or intraperitoneally, single or multiple injection, and with a wide range of concentrations. Whatever the regimen used, it is recommended that all MPTP injections to mice be performed in a fume hood. Vials from which MPTP is drawn should have a septum or be covered with parfilm to eliminate potential aerosols and spills and to avoid drops on the needle end. Change gloves frequently during the course of and at the end of the injection schedule. This will prevent any contamination of the PPE and decrease the possibility of overt contamination of equipment.

On the day of or on the evening before the experiment, all animals are weighed and coded. About a half-hour before starting the injection schedule, sterile MPTP solution should be prepared to the desired working concentration. During animal injection, care must be taken to avoid self-inoculation; special attention to animal restraint will significantly reduce this risk. For injection, place the mouse cage in the fume hood and when injecting, hold the animal so that any urine spray will fall into the cage and not on the surrounding areas, since mice, when held, tend to expel urine which can contain significant amounts of MPTP (Yang *et al.* 1988). Make sure the mouse is not held so tightly as to cause backflow of the injected MPTP from the peritoneum. Larger animals such as squirrel monkeys must be placed in restrainers for injection. It is not practical to inject large animals in a fume hood. Inspect injection site for leakage or spilled solution and wipe with a small pad dampened with 1% bleach solution. When discarding syringes, do not clip, recap or remove needles from syringes; fill the syringe with 1% bleach solution and then place the syringe with attached needle in a sharps container to be disposed of as biohazardous waste. At the end of the injection schedule, the remaining MPTP solution must be destroyed with an equivalent volume of 1% bleach solution as described above.

Cage changing

The greatest potential for exposure to MPTP and its metabolites is from contaminated bedding and caging immediately following MPTP injection and during the period that MPTP or its metabolites are likely to be in the excreta of treated animals. Therefore, when handling cages and their contents, it is important that the PPE be worn.

Used disposable mouse cages containing contaminated bedding should be dampened with 1% bleach solution and then be carefully placed into a plastic biohazard bag, tied off, and sent for incineration. When using re-usable cages, bedding should also be dampened with 1% bleach solution, then carefully placed in the biohazard bag, packaged and disposed of as biohazardous waste. Immediately after emptying re-usable cages, soak cages and accessories with 1% bleach solution for 10 min, rinse, then wash with detergent and rinse thoroughly with water. Mouse cages may then be sent to central cage washing facilities. The absorbent material that covered the rack surfaces should be sprayed with 1% bleach solution, allow to soak for 10 min and then disposed of as hazardous waste. For large animal cages, spray plastic-backed absorbent pads that line the cage bottoms and drop pans with 1% bleach solution, allow to soak for 10 min, then remove pads and place them in the biohazardous waste container; replace used linings with fresh pads. This needs to be done on a daily basis. Wash cages and accessories thoroughly with 1% bleach solution, rinse, then wash with detergent and rinse thoroughly with water. The procedure described above assumes that MPTP-injected animals remain in the same cage for 5 days postinjection and change out should occur only after the 5 days postinjection period. In the case of prolonged MPTP exposure protocols (i.e. weeks to months), while the procedure room or area will remain off-limits throughout the treatment period (plus the five days postinjection period), for mice, change only cage bottoms once a week following the procedure described above and, for monkeys, it is advisable to move monkeys to clean cages every other week and to handle the dirty cages as described above.

Counter tops in the procedure room or area should be cleaned with 1% bleach solution. Floor coverings should be carefully removed and disposed of as hazardous waste. Routine animal care can be re-instituted five days post last MPTP injection and once the procedure room or area has been cleaned by the responsible investigator and/or staff member.

Animal tissues

Potential risk of exposure to MPTP or MPP^+ may occur when animals are killed for tissue collection up to 5 days following MPTP administration. During this period, mice should be killed in the fume hood and the appropriate PPE worn by the researcher during blood and tissue harvesting procedures. All working surfaces are lined with plastic-backed absorbent pads, which should be changed if stained with body fluids. Since decapitation is the primary method of killing for small animals in MPTP studies, care should be taken to prevent blood spatters, and urine and feces should be contained. Brain tissues are best dissected on an inverted glass Petri dish covered with water-dampened filter paper and placed on regular ice. All instruments, including the Petri dish used for dissection, should be soaked in 1% bleach solution for 10 min, rinsed, then washed with detergent and rinsed with water. Collected tissues should always be handled with double gloves, and brain remnants and the remaining carcass, which may contain MPTP and

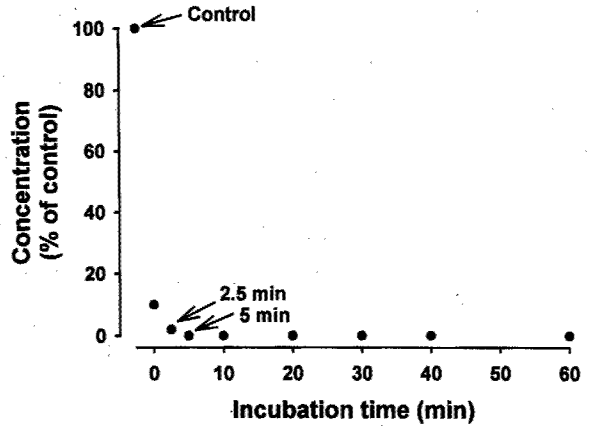

Fig. 1 Time-dependent effect of 1% bleach solution on MPTP. A 5-mg/mL of MPTP-HCl solution in saline was incubated at room temperature for different lengths of time with 1 volume (v/v) of 1% bleach (sodium hypochlorite) solution in water. After the indicated time of incubation, an aliquot of the mixture was injected into an HPLC-UV system and MPTP levels were quantified as described (Przedborski *et al.* 1996).

metabolites (Yang *et al.* 1988), must be discarded following biohazardous waste practices for animal waste.

For the perfusion of small animals, a grid overlaying a collection pan works best. Thus, blood and perfusion solution will be collected in the pan and can then be poured into a bottle or can be discarded as biohazardous waste. As per proper biohazardous waste disposal, the outside of the waste container must be wiped with 1% bleach solution.

For the perfusion of large animals, plastic tubing should be attached to the drain of the dissection table and a liquid biohazard waste container. This will catch any perfusion solution and prevent contamination of the water system. The collected perfusate will be discarded as biohazardous waste. After the perfusion procedure, the table must be washed with 1% bleach solution, rinsed, then washed with detergent and rinsed with water.

Decontamination, cleaning, and disposal

Often, one may see that 0.1 M HCl is used for cleaning up following MPTP experiments. However, we have HPLC evidence showing that HCl, up to 2 M and after incubation for more than 1 h at room temperature, does not destroy MPTP at all. Conversely, a 5% potassium permanganate solution in water completely destroys MPTP almost immediately. However, since potassium permanganate is such a powerful oxidant, it can produce hazardous exothermic reactions with several compounds like detergents and must be neutralized with ascorbic acid prior to being discarded as non-toxic waste. We have also found that bleach is as efficient as potassium permanganate in destroying MPTP, yet more friendly to use as it does not cause dangerous reactions with detergents and does not require specific treatment prior to discarding. Bleach is commercially available as a 5–10% stock solution. It can be readily diluted to the desired concentration with water and kept at room temperature indefinitely. Using a 1% bleach solution in water, which corresponds to twice the Environmental Protection Agency (EPA) recommended concentration for disinfection, we found that the action of bleach on 5 mg/mL of MPTP–HCl in saline is rapid in that after 5 min, at room temperature, there is no longer any detectable MPTP (Fig. 1). The 'almost' instantaneous destruction of MPTP by the bleach solution, as illustrated in Fig. 1, is not a surprising finding since the bleach-mediated reaction corresponds not to an enzymatic reaction but to a straight biochemical oxidation. In addition, we found that 10 min incubation of 5 mg/mL MPTP–HCl with different concentration of bleach solutions, ranging from 0.5 to 2.5%, had similar effects on MPTP. Therefore, our recommendation for MPTP decontamination is 10 min of soaking in 1% bleach solution. In contrast to their effects on MPTP, neither 2.5% bleach solution nor 5% potassium-permanganate destroyed MPP^+, even after an overnight incubation. This is not surprising, as MPP^+ is notoriously stable and resists destruction even after exposure to extremely harsh chemical and physical treatments. High doses of MPP^+ administered systemically (i.e. 25 mg/kg intraperitoneal) to mice produce oxidative damage to the lung, but fail to affect the nervous system (Johannessen *et al.* 1985). This is consistent with our observation that the intraperitoneal or subcutaneous injection of different doses of radiolabeled and non-radiolabeled MPP^+ to mice failed to show any accumulation of radioactivity in the striatum or to produce any damage to the dopaminergic systems of the brain (unpublished observation). Nevertheless, the direct injection of MPP^+ into the striatum does produce dopaminergic neurotoxicity (Giovanni *et al.* 1994b). These data indicate that the work-related hazards of MPP^+ involve peripheral organs such as the lungs and then only if high amounts reach the blood stream or the respiratory tract. Therefore, MPP^+ is far less hazardous than its parent compound and thus the real safety goal is the destruction of MPTP.

Only investigators appropriately trained in the handling of MPTP should clean up spills. Prior to any decontamination procedure, determine the maximum quantity of MPTP involved in the spill and the location of the spill.

If the room is properly maintained as stated above, linings and underpads will catch any spills. In case a liquid spill does occur, wearing the PPE, the researcher should immediately spray the linings and underpads with 1% bleach solution, allow to soak for 10 min, then remove, and place these in hazardous waste disposal bags. In the event that pads and linings have not caught all of the spill, absorb MPTP spill with absorbent plastic-backed pads to prevent

MPTP solution from contaminating gloves and discard as hazardous waste. The dry area is then soaked with 1% bleach solution, rinsed with water, then washed several times with detergent, rinsed with water, and dried with pads. Discard these materials in hazardous waste bags as well. Recover work area and inform the environmental health and safety office that an MPTP spill has occured and what measures were used to remove that spill.

To clean up MPTP powder spills, cover with a disposable towel dampened with 1% bleach solution, then pick up all materials and put into a hazardous waste container. Then, soak the area with 1% bleach solution, rinse with water, then wash several times with detergent, rinse with water, and dry with pads. Discard these materials in hazardous waste bags. Recover area, then inform the environmental health and safety office that a MPTP powder spill has occured and what measures were taken to contain and clean up the powder spill.

If clothes become contaminated with MPTP, immediately remove clothing and shower. After obtaining fresh clothing, report directly to a medical service. A very careful evaluation of any potential MPTP exposure is critical (see medical emergency and surveillance). Persons assisting exposed individuals should wear the PPE attire.

Plan experiments to avoid generating large quantities of contaminated glass or metal; these materials are difficult to incinerate, and large quantities can create waste disposal problems. Contaminated glass and metal can be decontaminated using 1% bleach solution followed by detergent washes and rinses. Decontaminate all equipment with wipes dampened with 1% bleach solution before repair work is performed, before transferring equipment to other operations, and before discarding. Pay special attention to internal parts of equipment that may have become contaminated.

Prevention, medical emergency and surveillance

To date, there has been no report in the literature of the inadvertent exposure of a researcher to MPTP while conducting MPTP experiments. A single report of a research chemist who suffered a fatal exposure to large amounts of MPTP during its synthesis has been documented and represents the only inadvertent human exposure to MPTP (Langston and Ballard 1983). However, despite the safe track record of MPTP use, precautionary emergency procedures must be employed to avoid potential injury from acute exposure to the toxin (such as a needle prick).

As indicated above, MAO-B inhibitors prevent the conversion of MPTP to its toxic metabolite, MPP^+ thereby preventing neurotoxicity. For example, pretreatment of animals with deprenyl, a potent irreversible MAO-B inhibitor prevents MPTP-induced neurotoxicity (Cohen *et al.* 1984; Mytilineou and Cohen 1985; Fuller *et al.* 1988). On the other hand, except for a single report (Tatton 1993), there is no evidence that MAO-B inhibition by deprenyl or by other compounds, following exposure to MPTP provides any neuroprotection. However, in case of accidental exposure to MPTP, in an attempt to block the conversion of any remaining MPTP to MPP^+ it is recommended that deprenyl be administered immediately. As far as we know, there is no established deprenyl regimen for accidental exposure to MPTP. Since the goal here is to prevent the conversion of MPTP by inhibiting MAO-B, as rapidly and profoundly as possible, we suggest an initial large dose of deprenyl (e.g. four 5 mg tablets) be taken orally at once. Although it may be prudent to continue deprenyl medication (e.g. 5 mg twice a day) for some time, it is unknown whether this is justified. Short-term surveillance is necessary for the appearance of hypotension from the deprenyl or the development of acute parkinsonian symptoms from the MPTP exposure. In addition, following the administration of a large dose of deprenyl, individuals must be cautious in consuming tyramine-containing foods (i.e. cheese) and in taking medications containing pharmacologically active amines. Prior to beginning any MPTP investigation, deprenyl must be available for emergency use and must be kept in a closed container at all times in the procedure room or area for immediate use, if necessary. Furthermore, it is advisable that individuals who are planning to embark upon a series of MPTP experiments consider a treatment of 5 mg twice a day of deprenyl prior to (e.g. 3–5 days before) and during the experiments. This may be especially indicated for a person first learning the protocol or if there is an increased risk of contact with MPTP. This should be done only after consulting one's personal physician.

Conclusion

To date, MPTP remains the best experimental model of PD. To this end, it is extensively used in various animal species and especially in mice. However, even as a research tool, MPTP is an extremely hazardous compound, which can be injected, ingested, inhaled, and/or absorbed. Because of its demonstrated toxicity to humans, the use of MPTP among researchers is a serious concern. Over the years, a better understanding of the physicochemical properties of this toxin, its metabolism, and its body distribution has enabled investigators to develop practices and procedures for the safe use of this compound. These include improved procedures for preparing MPTP solutions and for its injection into animals, proper protective equipment, reducing potential exposure from animal excreta, proper decontamination and disposal procedures, and medical treatment and surveillance in case of accidental exposure. Despite the fact that we have tried to cover the most common situations and topics related to MPTP use, this review cannot cover all possible aspects of the safe use of

this hazardous compound. Accordingly, there can be no substitute for common sense and proper laboratory practices in the use of dangerous compounds such as MPTP. It is hoped, however, that this review has built upon the guidelines presented by others in the past and, in conjunction with our recent knowledge of MPTP, will lead to the effective and safe use of the MPTP animal model of PD.

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Bax ablation prevents dopaminergic neurodegeneration in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease

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1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) damages dopaminergic neurons in the substantia nigra pars compacta (SNpc) as seen in Parkinson's disease. Here, we show that the pro-apoptotic protein Bax is highly expressed in the SNpc and that its ablation attenuates SNpc developmental neuronal apoptosis. In adult mice, there is an up-regulation of Bax in the SNpc after MPTP administration and a decrease in Bcl-2. These changes parallel MPTP-induced dopaminergic neurodegeneration. We also show that mutant mice lacking Bax are significantly more resistant to MPTP than their wild-type littermates. This study demonstrates that Bax plays a critical role in the MPTP neurotoxic process and suggests that targeting Bax may provide protective benefit in the treatment of Parkinson's disease.

Parkinson's disease (PD) is a common neurodegenerative disorder whose cardinal clinical features include tremor, slowness of movement, stiffness, and postural instability (1). These disabling symptoms are primarily due to a profound deficit in striatal dopamine content that results from the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the consequent loss of their projecting nerve fibers in the striatum (2, 3). Although several approved drugs do alleviate PD symptoms, their chronic use often is associated with debilitating side effects (4), and none seem to dampen the progression of the disease. Moreover, the development of effective neuroprotective therapies is impeded by our limited knowledge of the mechanism by which SNpc dopaminergic neurons die in PD. Thus far, however, significant insights into the pathogenesis of PD have been achieved by the use of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which replicates in humans and nonhuman primates a severe and irreversible PD-like syndrome (5). In several mammalian species, MPTP reproduces most of the biochemical and pathological hallmarks of PD, including the dramatic degeneration of dopaminergic neurons (5).

Mounting evidence indicates that highly regulated cell death-associated molecular pathways could participate in the relentless demise of neurons in degenerative diseases (6, 7), including PD (8). In keeping with this, Bax (9) has emerged as a pro-cell death driving force within the central decision point constituted by the Bcl-2 family that modulates the activation of downstream effectors of cell death such as caspases (7). It is also clear that Bax is required for the death of several types of neurons in the peripheral and central nervous systems during both normal development and pathological situations (10–18). In light of its critical role within the programmed cell death machinery and its importance in neuronal death, Bax appears as a particularly appealing target for therapeutic interventions aimed at hampering neurodegeneration. Consistent with a potential pivotal role for Bax in SNpc neuronal death, here we show that Bax is highly expressed in the SNpc and that its ablation attenuates SNpc developmental neuronal apoptosis. We demonstrate that there is a dramatic up-regulation of Bax mRNA and protein in the SNpc of adult mice after MPTP administration. These changes parallel the time course of MPTP-induced dopaminergic neurodegeneration. We also show that mutant mice lacking Bax are resistant to MPTP compared with their wild-type littermates, thus indicating that Bax is a key factor in MPTP-induced SNpc dopaminergic neurodegeneration.

Materials and Methods

Animals and Treatment. C57/bl mice heterozygous for Bax were mated to yield F_1 offspring with $Bax^{-/-}$, $Bax^{+/-}$, and wild-type genotypes. Tail DNA was prepared and screened for both the normal and the mutant allele by using a single PCR. The normal allele was amplified by using an exon 5 forward primer (0.64 μM: 5′-TGATCAGAACCATCATG-3′) and an intron 5 reverse primer (0.64 μM: 5′-GTTGACCAGAGTGGCGTAGG-3′), which together generated a 304-bp product. The mutant allele was amplified with a neo/pgk primer (0.16 μM: 5′-CCGCTTCCATTGCTCAGCGG-3′) and the same intron 5 reverse primer, which together generated a 507-bp product. Cycling parameters were 1 min at 94°C, 55°C, and 72°C each for a total of 30–35 cycles. The primer ratio was adjusted to allow amplification of both products simultaneously with preferential amplification of the wild-type allele to assure correct genotyping of the Bax-deficient animals. All mice used in this study were treated according to National Institutes of Health guidelines for Care and Use of Laboratory Animals and with the approval of Columbia University's Institutional Animal Care and Use Committee. Eight-week-old male mice received one i.p. injection of MPTP-HCl per day (30 mg/kg per day of free base; Research Biochemicals, Natick, MA) for 5 consecutive days and were killed at 0, 2, 4, 7, 21, and 42 days after the last injection; control mice received saline injections only. Both saline and MPTP animals then were divided into two groups. The first group was perfused and brains were used for immunohistochemistry, whereas the second group of mice were killed, and brains were quickly removed, dissected (midbrain, striatum, cerebellum, and

Abbreviations: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; SNpc, substantia nigra pars compacta; PD, Parkinson's disease; TH, tyrosine hydroxylase; QA, quinolinic acid; MPP^+, 1-methyl-4-phenylpyridinium ion.

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cortex), snap-frozen on dry ice, and stored at −80°C for Western blot, immunoprecipitation, and reverse transcriptase–PCR analysis. MPTP use and safety precautions were as described (19).

Immunohistochemistry and Double Immunofluorescence. Immunohistochemistry was performed as described by Vila *et al.* (20) with a polyclonal antibody to Bax (1:500; polyclonal; PharMingen). Immunostained sections then were counterstained with thionin. To examine the colocalization of Bax with tyrosine hydroxylase (TH), a double immunofluorescence technique was performed by using the same polyclonal anti-Bax antibody (1:200 dilution) and a monoclonal antibody to TH (1:200 dilution; Boheringer Mannheim). Sections were examined on green, red, and double (green + red) filters by using confocal microscopy.

Striatal Lesions with Quinolinic Acid (QA). After Metofane inhalation, mouse pups aged postnatal day seven of either sex received an intrastriatal injection of 0.5 μl of a 480 nmol solution of QA dissolved in PBS at pH 7.4 as described (21). One day after the QA injection, animals were perfused and brains were processed for morphological analysis.

Immunoblots and Immunoprecipitation. For Western blot analysis, total tissue proteins were isolated in 50 mM Tris·HCl, pH 7.0/150 mM NaCl/5 mM EDTA/1% SDS/1% Nonidet P-40/protease inhibitors (Mini mixture; Roche Diagnostics, Indianapolis, IN). Incubation with primary antibody was performed overnight at 4°C with monoclonal antibodies to Bax (1:1,500 dilution; Santa Cruz Biotechnology) or Bcl-2 (1:500 dilution, Transduction Laboratories, Lexington, KY) and, as an internal control, a monoclonal antibody to β-actin (1:5,000, Sigma). Films were quantified by using the National Institutes of Health IMAGE analysis system. For immunoprecipitation, frozen samples from saline-injected mice and MPTP-intoxicated animals (at day 4 after the last MPTP injection) were homogenized in 10 vol (wt/vol) of 10 mM Hepes (pH 7.20) containing 0.25% Nonidet P-40, 142.5 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, and one tablet of protease inhibitor mixture. Then, 250 μg of protein was incubated (overnight, 4°C) with 3 μg of a polyclonal antibody to Bcl-2 (N-19, Santa Cruz Biotechnology) and further processed for immunoprecipitation and immunoblotting as described by Ara *et al.* (22). Here, blots were immunostained with either a monoclonal antibody to Bax (1:1,500 dilution; Santa Cruz Biotechnology) or a monoclonal antibody to Bcl-2 (1:1,000 dilution; Transduction Laboratories).

RNA Extraction and Reverse Transcriptase–PCR. Total RNA was extracted from midbrain, striatal, and cerebellar samples from saline and chronic MPTP-treated animals and used for reverse transcriptase–PCR analysis as described by Vila *et al.* (20). The Bax primer sequences were 5′-CTGAGCTGACCTTGGAGC-3′ (forward) and 5′-GACTCCAGCCACAAAGATG-3′ (reverse). As an internal control, β-actin cDNA was coamplified by using primer sequences 5′-CTTTGATGTCACGCACGATTTC-3′ (forward) and 5′-GGGCCGCTCTA GGCACCAA-3′ (reverse). Each PCR cycle consisted of denaturation at 94°C for 5 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min, followed by a final 10-min extension at 72°C. PCR amplification was carried out for 30 cycles for Bax and 22 cycles for β-actin by using a Perkin–Elmer GeneAmp 9700 Thermal Cycler.

Stereology and Quantification of Apoptotic Neurons. The total number of TH-positive SNpc neurons was counted in the different groups of animals at 21 days after the last MPTP or saline injection by using the optical fractionator method as described by Liberatore *et al.* (23). This unbiased method of cell counting is not affected by either the volume of reference (SNpc) or the size of the counted elements (neurons). Immunostaining was performed with a polyclonal antibody to TH (1:1,000; Calbiochem), and sections were counterstained with thionin. Quantification of the number of apoptotic neurons in the SNpc of MPTP- and saline-injected mice was assessed as described (21). Morphological criteria to identify apoptotic figures included shrinkage of cellular body, chromatin condensation, and the presence of distinct, round, well-defined chromatin clumps, demonstrated by thionin staining (21).

Measurement of Striatal Dopamine, 3,4-Dihydroxyphenylacetic Acid, and Homovanillic Acid Levels. HPLC with electrochemical detection was used to measure striatal levels of dopamine, 3,4-dihydroxyphenylacetic acid, and homovanillic acid by using a method that has been described by Przedborski *et al.* (24), with minor modifications of the mobile phase. At 21 days after the last MPTP injection, animals were killed and striata were dissected out and processed for HPLC measurement. The modified mobile phase consisted of 0.15 M monochloroacetic acid, pH 3.0, 200 mg/liter sodium octyl sulfate, 0.1 mM EDTA, 4% acetonitrile, and 2.5% tetrahydrofuran.

Measurement of Striatal MPP^+ Levels. HPLC with UV detection (wavelength, 295 nm) was used to measure striatal MPP^+ levels as described by Przedborski *et al.* (24). Groups of $Bax^{+/-}$, $Bax^{-/-}$, and wild-type littermates were killed at 90 and 180 min after one i.p. injection of 30 mg/kg MPTP, and the striata were dissected and processed for HPLC.

Statistical Analysis. All values are expressed as the mean ± SEM with time, treatment, or genotype as the independent factors. When ANOVA showed significant differences, pair-wise comparisons between means were tested by Newman-Keuls post hoc testing. In all analysis, the null hypothesis was rejected at the 0.05 level.

Results

High Expression of Bax in SNpc Dopaminergic Neurons. Relevant to the potential role of Bax in PD neurodegeneration, we found that virtually all neurons of the SNpc exhibit conspicuous levels of Bax protein, as evidenced by immunohistochemistry (Fig. 1*a*). SNpc neurons are primarily dopaminergic and secondarily GABAergic (25). Thus, to confirm that dopaminergic neurons do contain Bax protein, we performed double immunohistochemistry for TH, the rate-limiting enzyme in dopamine synthesis, and Bax. Examination by confocal microscopy demonstrated that all TH-positive neurons expressed Bax (Fig. 1 *b* and *c*) and, as expected given the ubiquitous expression of Bax in the brain, that Bax was expressed by both TH-positive and TH-negative neurons. Most Bax-positive SNpc dopaminergic neurons showed a prominent punctate immunoreactivity superimposed onto a diffuse cytoplasmic immunostaining (Fig. 1*d*), which is consistent with the known subcellular distribution of Bax in both mitochondria and cytosol (9, 26).

Bax Modulates Developmental Cell Death in the SNpc. During development, neurons in the SNpc undergo an intense naturally occurring cell death process (21, 27). Dying neurons exhibit the morphological characteristics of apoptosis and their numbers are modulated by the size of striatum (21, 27), the brain structure in which SNpc neuron projections form synapses. Indeed, 24 h after unilateral destruction of the striatum with a local injection of the excitotoxin QA at postnatal day seven, wild-type pups showed four times more apoptotic neurons in the SNpc ipsilateral to the lesion compared with the contralateral side (Fig. 2). Age-matched mutant pups heterozygous ($Bax^{+/-}$) or homozygous ($Bax^{-/-}$) for the *Bax* null mutation showed a gene dosage-

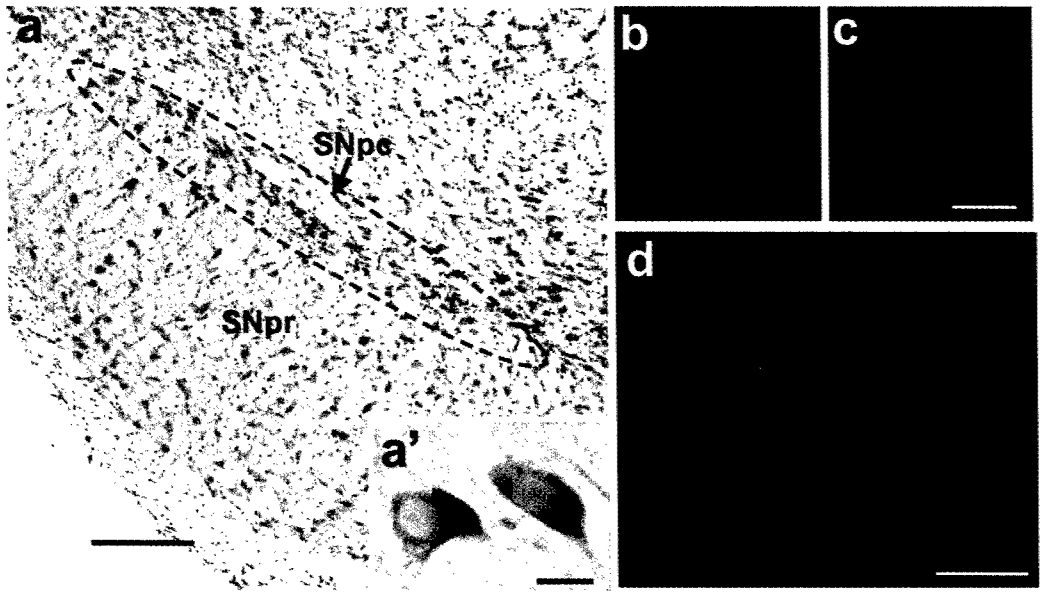

Fig. 1. Bax expression in SNpc dopaminergic neurons of adult mice. (*a*) Bax is highly expressed in SNpc neurons, as assessed by immunohistochemistry; sections are counterstained with thionin. (*a'*) High magnification of Bax-immunostained neurons in the SNpc. (*b* and *c*) Double immunofluorescence with antibodies to Bax and TH confirms that Bax (in green) is expressed in dopaminergic neurons (in red). (*d*) Confocal microscopy analysis of Bax-positive dopaminergic neurons (Bax + TH immunostaining) shows a robust punctate immunoreactivity superimposed onto a diffuse cytoplasmic immunostaining. [Scale bars: 200 μm (*a*), 10 μm (*a'* and *d*), and 30 μm (*b* and *c*).]

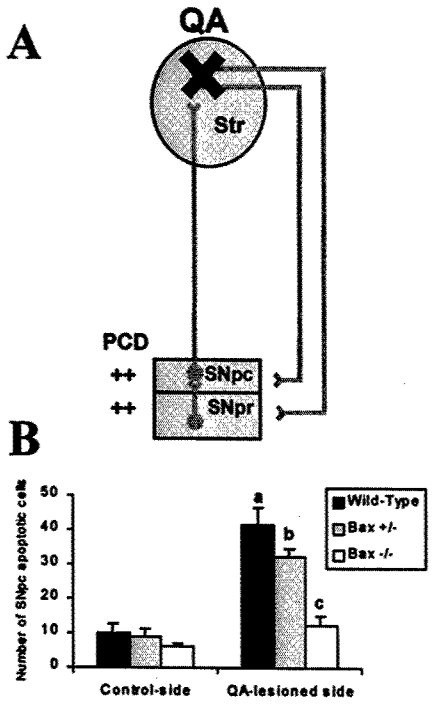

Fig. 2. Bax regulates natural and QA-induced developmental neuronal death in the SNpc. (*a*) Schematic representation of the model of induced apoptotic cell death in the SNpc by unilateral destruction of the striatum (i.e., the target) at postnatal day seven with a local injection of QA. (*b*) 24 h after the lesion, wild-type mice (n = 5) exhibit a substantial number of dying neurons with a definite morphology of apoptosis in the contralateral SNpc and a dramatic increase in this number in the SNpc ipsilateral to the QA lesion. Age-matched mutant mice deficient for Bax ($Bax^{+/-}$ and $Bax^{-/-}$, n = 4 per group) exhibit a striking lower number of SNpc apoptotic neurons after QA administration. *a*, $P < 0.05$, compared with wild-type control-side; *b*, $P < 0.05$, compared with $Bax^{+/-}$ control-side; *c*, $P < 0.05$, compared with wild-type and $Bax^{+/-}$ QA-lesioned sides but not significant when compared with $Bax^{-/-}$ control side; Newman-Keuls post hoc analysis.

dependent reduction of SNpc apoptotic neurons after QA administration (Fig. 2).

MPTP Stimulates Bax Expression in Ventral Midbrain. In saline-injected mice, there was a high constitutive expression of Bax protein in the ventral midbrain (Fig. 3*a*). After systemic MPTP administration, there was a dramatic up-regulation of Bax protein in this brain region (Fig. 3*a*), in agreement with a previous study (28). This change occurred in a time-dependent manner, with protein levels peaking at 4 days after the last MPTP injection (+668%), then progressively returning to control levels (Fig. 3*a*). This alteration was not only time-dependent but was also region-specific as MPTP-intoxicated mice showed no Bax up-regulation, at any of the time points studied, in striatum or in cerebellum, two brain regions devoid of neuronal loss after MPTP administration.

MPTP Increases Bax mRNA Levels in Ventral Midbrain. Given the change in Bax protein after MPTP injections, we also investigated whether this change was associated with Bax transcriptional alterations. In saline-injected mice, there was a constitutive level of Bax transcript in the ventral midbrain (Fig. 3*b*). In MPTP-injected mice, there was a time-dependent increase in the level of Bax transcript, which peaked at 2 days after the last MPTP injection (+364%), then progressively returned to the level of controls by day 7 (Fig. 3*b*). Bax mRNA up-regulation was also region-specific as it was not detected in the striatum nor in the cerebellum of MPTP-intoxicated animals.

Time Course of MPTP-Induced Apoptotic Neuronal Death. Quantification of apoptotic cells in the SNpc of MPTP- and saline-injected mice indicates that apoptotic neuronal death culminated between days 2 and 4 after the last MPTP injection (Fig. 3). Morphological criteria used to identify apoptotic cells were previously validated (21) and included shrinkage of cellular body, chromatin condensation, and presence of distinct, round, well-defined chromatin clumps, demonstrated by thionin staining.

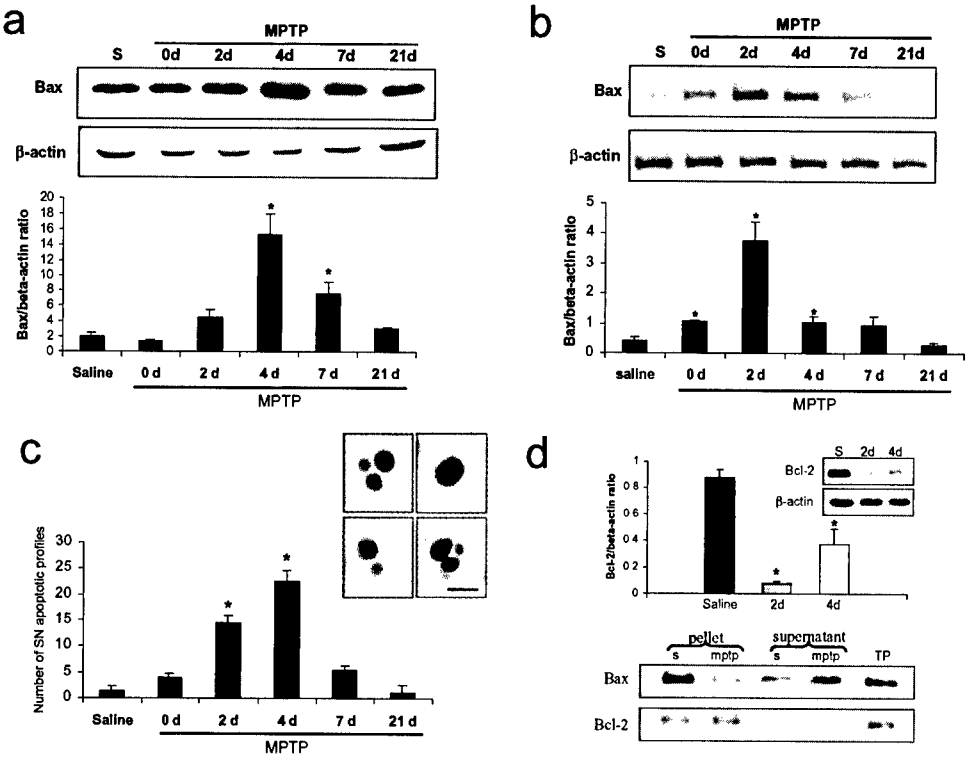

Fig. 3. Bax expression in the ventral midbrain after MPTP intoxication. (*a*) Bax protein levels in the ventral midbrain ($n = 3$ mice per group) were assessed by Western blot analysis. (*b*) Bax mRNA expression in the ventral midbrain was quantified by reverse transcriptase–PCR ($n = 3$–5 mice per group). (*c*) Bax protein and mRNA up-regulation coincide with the time course of apoptotic-induced cell death in the SNpc. Morphological criteria to identify apoptotic figures, as illustrated in photomicrographs, included shrinkage of cellular body, chromatin condensation, and the presence of distinct, round, well-defined chromatin clumps, demonstrated by thionin staining. (Scale bar, 5 μm.) (*d*) Bcl-2 protein expression (*Upper*) and immunoprecipitation (*Lower*) after MPTP intoxication. Bcl-2 protein levels are decreased in the ventral midbrain of MPTP-intoxicated mice at days 2 and 4 after the last MPTP injection ($n = 3$–5 mice per group). At day 4 after the last injection, ventral midbrain proteins ($n = 4$ mice per group) were subjected to immunoprecipitation with a polyclonal antibody to Bcl-2. The amount of Bax coimmunoprecipitated with Bcl-2 appeared less abundant in the pellets of MPTP-intoxicated mice than in those of saline-injected animals. This was associated with increased Bax immunoreactivity in the supernatant. S, saline; TP, total proteins. *, $P < 0.05$, compared with saline-injected animals; Newman-Keuls post hoc analysis. Error bars indicate SEM.

MPTP Decreases Bax:Bcl-2 Heterodimerization in the Ventral Midbrain. Several members of the Bcl-2 family, such as Bcl-2, can bind to Bax to form Bax:Bcl-2 heterodimers, hence antagonizing Bax pro-cell death properties (9). Accordingly, we determined the levels of Bcl-2 protein as well as its capacity to heterodimerize with Bax protein in ventral midbrain of MPTP-intoxicated mice, at the peak of MPTP-induced apoptotic neuronal death. In striking contrast with Bax up-regulation, Bcl-2 protein levels, as assessed by Western blot, were dramatically decreased in ventral midbrain of MPTP-intoxicated mice compared with saline-injected animals, 2 and 4 days after the last injection (Fig. 3*d*). Furthermore, the amount of Bax that coimmunoprecipitated with Bcl-2 at this time point, using an anti-Bcl-2 antibody, was much less in MPTP-intoxicated mice than in saline-injected animals (Fig. 3*d*). Consistent with this finding, the amount of Bax that escaped coimmunoprecipitation using an anti-Bcl-2 antibody was much greater in MPTP-intoxicated mice than in saline-injected animals (Fig. 3*d*). The ratio of these proteins indicates that most of the Bax protein could be inactivated by Bcl-2 in saline-injected mice whereas there is an excess of unopposed Bax in MPTP-injected mice.

Bax-Deficient Mice Are Resistant to MPTP Intoxication. To confirm the involvement of Bax in MPTP-induced neuronal death, we compared the effects of MPTP in $Bax^{+/-}$ and $Bax^{-/-}$ mice and in their wild-type littermates. In saline-injected mice, no significant changes in stereological counts of SNpc dopaminergic neurons, defined by TH immunostaining, were detected between the different groups of mice (Fig. 4*A*). In wild-type mice, MPTP caused a dramatic loss of SNpc TH-positive neurons, which was accompanied by a large number of apoptotic neurons (Fig. 4 *A* and *B*). MPTP can down-regulate phenotypic markers such as TH (29), thus it is important to indicate that the TH/Nissl ratio of neuronal counts did not differ between saline- and MPTP-injected wild-type mice (saline = 1.78 ± 0.05 vs. MPTP = 1.77 ± 0.03; $n = 3$ per group; Student's t test), confirming that the reduction in TH-positive neurons corresponds to an actual loss of neurons. In contrast to the situation in wild-type animals, MPTP failed to affect SNpc TH-positive neuronal counts in $Bax^{-/-}$ and caused only a mild reduction of these numbers in $Bax^{+/-}$ mice (Fig. 4*A*). Similarly, the number of MPTP-induced SNpc apoptotic neurons was significantly smaller in $Bax^{-/-}$ and, to a lesser extent, in $Bax^{+/-}$ than in wild-type animals (Fig. 4*B*). Although less striking than the loss of the SNpc cell body counts, the loss in striatal dopaminergic nerve terminals after MPTP administration, as assessed by measuring the levels of dopamine and its two main metabolites 3,4-dihydroxyphenylacetic acid and homovanillic acid, was also markedly attenuated in $Bax^{-/-}$ and $Bax^{+/-}$ mice, compared with their wild-type littermates (Table 1).

MPP^+ Production in Bax-Deficient Mice. The main determining factor of MPTP neurotoxic potency is its conversion in the brain to 1-methyl-4-phenylpyridinium ion (MPP^+) (30). To confirm that the resistance of $Bax^{-/-}$ mice is due to the absence of the *Bax* gene and not to an alteration in the brain's production of

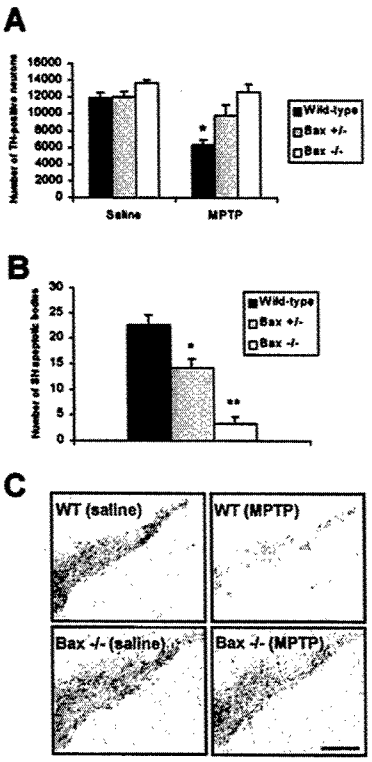

Fig. 4. Bax-deficient mice are resistant to MPTP neurotoxic effect. (*A*) Stereological counts of TH-positive neurons in the SNpc were performed in Bax-deficient mice and their wild-type littermates at day 21 after the last injection (n = 3–5 mice per group). In wild-type mice, only 53% of the SNpc TH-positive neurons survived MPTP administration. In contrast, 81% of SNpc TH-positive neurons survived in $Bax^{+/-}$ mice and no loss of TH-positive cells was found in $Bax^{-/-}$ animals under an identical MPTP regimen. *, $P < 0.05$, compared with saline-injected wild-type animals; Newman-Keuls post hoc analysis. (*B*) At the peak of apoptotic cell death (day 4 after the last MPTP injection), $Bax^{-/-}$ mice (n = 3) presented 85% reduction in the number of apoptotic profiles in the SNpc compared with MPTP-intoxicated control animals (n = 4). In $Bax^{+/-}$ animals (n = 4), these numbers were reduced by 37%. *, $P < 0.05$ compared with MPTP-intoxicated control animals; **, $P < 0.05$ compared with MPTP-intoxicated control animals and MPTP-intoxicated $Bax^{+/-}$ mice; Newman-Keuls post hoc analysis). Error bars indicate SEM. (*C*) Photomicrographs of TH-immunostained sections with thionin counterstain, illustrating the results in *A*. (Scale bar, 400 μm.)

MPP^+, we measured striatal content of MPP^+ at different time points after MPTP administration. At no time point does the striatal content of MPP^+ differ significantly among $Bax^{+/-}$, $Bax^{-/-}$, and wild-type littermate mice (Table 2).

Table 1. Striatal monoamine levels (ng/mg tissue)

Mice	Dopamine	DOPAC	HVA
Saline			
Wild type	12.2 ± 0.2	2.1 ± 0.2	1.8 ± 0.1
$Bax^{+/-}$	13.3 ± 0.3	2.0 ± 0.1	1.7 ± 0.1
$Bax^{-/-}$	13.4 ± 0.4	2.4 ± 0.1	1.6 ± 0.2
MPTP			
Wild type	0.5 ± 0.1	0.3 ± 0.05	0.2 ± 0.01
$Bax^{+/-}$	3.1 ± 0.3*	0.6 ± 0.06	0.6 ± 0.04*
$Bax^{-/-}$	4.1 ± 0.2**	1.3 ± 0.2**	1.0 ± 0.1**

DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid. *, $P < 0.05$, compared to MPTP-injected wild-type mice; **, $P < 0.05$, compared to MPTP-injected wild-type and MPTP-injected $Bax^{+/-}$ mice; Newman-Keuls post hoc test. Data represent means ± SEM for 4–6 mice per group.

Table 2. Striatal MPP^+ levels (μg/g striatum) in Bax-deficient and wild-type mice

Mice	90 min	180 min
Wild type	8.5 ± 1.0	5.7 ± 0.9
$Bax^{+/-}$	7.6 ± 1.9	5.1 ± 0.3
$Bax^{-/-}$	10.0 ± 1.0	5.4 ± 0.8

HPLC measurements of striatal MPP^+ levels in wild-type and Bax-deficient mice were determined at 90 and 180 min after a single i.p. MPTP injection (30 mg/kg). n = 4 animals per group. Values represent the mean ± SEM.

Discussion

Bax is widely expressed in the central nervous system, where it is detected primarily in neurons (9, 31). Herein, we show that almost all neurons of the SNpc, especially all dopaminergic neurons, contain abundant amounts of Bax protein (Fig. 1), likely located both at mitochondria and in cytosol (9). We also demonstrate that Bax controls the apoptotic demise of SNpc dopaminergic neurons during development, because its ablation attenuates SNpc developmental cell death in immature animals (Fig. 2). These findings confirm a key role for Bax in the fate of SNpc neurons, thus setting the stage for Bax being a potential culprit in the degeneration of SNpc dopaminergic neurons in PD.

To test the contribution of Bax in PD neurodegeneration, we used the experimental model produced by the parkinsonian neurotoxin MPTP (5). Because the mode of cell death in PD may be, at least in part, apoptotic (8), we selected a MPTP regimen that kills SNpc dopaminergic neurons by apoptosis (32). This regimen induces a time-dependent apoptotic cell death in the SNpc that is maximal between 2 and 4 days after the last dose of MPTP (Fig. 3*c*). Relevant to the known pro-apoptotic role of Bax, we found that the time course of SNpc apoptotic neuronal death coincides with that of increased levels of Bax mRNA and protein in ventral midbrain after MPTP administration (Fig. 3 *a* and *b*). The opposite image was found for Bcl-2 in that at 2 and 4 days post-MPTP ventral midbrain Bcl-2 protein levels were markedly reduced. These findings suggest that, during the MPTP-induced neurodegenerative process, the finely tuned balance between cell death agonists, such as Bax, and cell death antagonists, such as Bcl-2, is upset in the ventral midbrain, leading to a situation in which molecular pro-apoptotic forces dominate (33). In this context, an aspect related to Bax function is its capacity to form heterodimers with Bcl-2 and homomultimers with itself (34). In saline-injected mice, the amount of Bax can theoretically be neutralized by Bcl-2 as evidenced by the majority of Bax in heterodimers. Whereas, in MPTP-injected mice excess Bax exists free of neutralizing interaction with Bcl-2 (Fig. 3*d*). Taken together, our data suggest that, after MPTP administration, a cascade of deleterious events is set in motion within which Bax up-regulation and Bcl-2 down-regulation are key factors. Consistent with this scenario, the observed neuroprotective effects provided by Bcl-2 overexpression against MPTP (35, 36) may reflect its capacity to counter Bax.

Consistent with the involvement of Bax in the MPTP neurotoxic process is our demonstration that no significant loss of SNpc dopaminergic neurons was observed in $Bax^{-/-}$ mice and that approximately 81% of SNpc dopaminergic neurons survived in $Bax^{+/-}$ mice compared with their wild-type littermates after MPTP administration (Fig. 4). Similarly, there were significantly fewer apoptotic neurons in the SNpc of $Bax^{+/-}$ and $Bax^{-/-}$ after MPTP administration compared with wild-type controls (Fig. 4). The resistance of the SNpc dopaminergic neurons in Bax knockout mice was accompanied by a significant, although less prominent, sparing of striatal dopamine contents (Table 1). The latter suggests that Bax ablation protects against SNpc neuronal death, but still allows some changes in gene expression and/or alter-

ations in dopamine synthesis. Relevant to this, is our previous demonstration that TH, the rate-limiting enzyme in dopamine synthesis, is inactivated by tyrosine nitration after MPTP administration (22).

We also found that ablation of Bax was not associated with alterations in the formation of MPTP active metabolite, MPP^+ (Table 2), which is the most significant modulating factor of MPTP potency (30).

In light of the results reported above, including the resistance of Bax-deficient mice to the neurotoxic effects of MPTP, we argue that Bax is a critical effector molecule in MPTP-mediated cell death. Given the mode of action of MPTP and Bax, it is possible that the mitochondrion is key to the observed neuroprotection. Models of Bax activation indicate its oligomerization may result in a homomultimeric pore (37), a VDAC-containing pore (38), or a permeabilization of mitochondrial outer membrane (39) to release cytochrome *c*. Several lines of evidence indicate that translocation of mitochondrial cytochrome *c* to the cytosol is a critical event in the mitochondrial-dependent activation of effector caspases such as caspase-3 and ensuing cell death (40). Providing credence to this proposed sequence of events in PD is the observation that caspase-3 is indeed activated in postmortem SNpc samples from parkisonian patients (41). Once inside dopaminergic neurons, MPP^+ is actively concentrated within mitochondria, where it inhibits complex I of the electron transport chain (5). This inhibition leads to a deficit in ATP formation and to an increase in reactive oxygen species production (5), which, in turn, cause an energy crisis and oxidative stress. As with other situations, mitochondrial dysfunction seen after MPTP administration ultimately can trigger large amplitude swelling often attributed to the opening of the permeability transition pore complex (PTPC). Alternatively, the opening of the PTPC can lead to several dramatic consequences, including a dissipation of the mitochondrial transmembrane potential and a release to the cytosol of proteins normally confined to the mitochondria, such as cytochrome *c* (42).

Collectively, our results indicate that Bax plays a pivotal role in SNpc dopaminergic neuronal death in the MPTP mouse model likely by acting in injured neurons before the onset of irreversible cell death events. Whether blocking events downstream of Bax also can protect these cells remains to be determined. Ablation of the cell executioner, caspase-3, dramatically decreases neuronal death during development (43). However, whether inhibition of caspases downstream of mitochondria will prove sufficient to interfere with adult-onset pathological stimuli or merely shift the mode of death of severely injured neurons remains uncertain. Because of the striking similarities between the MPTP model and PD, the present study raises the possibility that Bax plays a critical role in the neurodegenerative process of PD and thus that targeting Bax could open new neuroprotective avenues for this disabling neurological disease.

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Free Radicals in Brain Pathophysiology

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ROS and Parkinson's Disease: A View to a Kill

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I. INTRODUCTION

Under normal and pathological conditions, cellular metabolism generates substantial amounts of free radicals, i.e., atoms and molecules with one or more unpaired electrons (1). By virtue of this electron imbalance, free radicals are unstable and prone to snatch electrons from neighboring atoms and molecules, whereby they inflict oxidative damage. The classical view is that free radical–induced oxidative damage of biological compounds such as DNA, proteins, and lipids may cause serious cellular derangement and ultimately even cell death. Of note, not all atoms or molecules that can cause oxidative damage are free radicals because they do not have unpaired electrons (e.g., singlet oxygen, hydrogen peroxide). Therefore, those species that either have or do not have unpaired electrons and are all mainly oxygen-centered we refer to as reactive oxygen species (ROS).

For the past decade, the oxidative stress hypothesis has gained in popularity to explain cell death in neurological diseases as diverse as stroke, dementia, amyotrophic lateral sclerosis, and Parkinson's disease (PD). The latter disorder is viewed by some as the model "par excellence" of oxidative stress in chronic neurological conditions for several reasons that will be reviewed and discussed below. However, while compelling evidence exists to implicate ROS in the pathogenesis of acute neurological conditions, such as strokes, the jury is still out as to whether the role of ROS in chronic neurodegenerative disorders such as PD is myth or reality. In addition, even if one believes the latter, one is left with the dilemma as to whether the oxidative damage observed in PD brains (detailed

below) is the cause or the consequence of the neurodegenerative process, or is the result of the chronic use of anti-PD treatments such as levodopa. These various issues will be discussed in light of earlier landmark studies, as well as of more recent findings obtained in humans, in animals, and in cell culture.

II. PARKINSON'S DISEASE

Parkinson's disease affects about 1% of the population over 50 years of age in the United States alone; about 50,000 new cases are diagnosed each year (2). This common neurodegenerative disorder, which is mainly sporadic, is a slow, progressive disease characterized mainly by resting tremor, slowness of movement (bradykinesia), stiffness (rigidity), and poor balance (postural instability) (2). Most if not all of these symptoms are attributed to the severe loss of dopamine (DA)–containing neurons in the substantia nigra pars compacta (SNpc) and the concomitant loss of DA nerve terminals in the caudate putamen, which is the main projection area for the SNpc neurons (3). To a lesser extent, neuronal loss is found in the locus coeruleus and in the dorsal motor nucleus of vagus (3). Another morphological hallmark of PD is the eosinophilic intraneuronal inclusion called the Lewy body (4), which is regarded as either a tombstone for the cell or a key player in the neurodegenerative process. PD patients can avoid medication for a while, but at some point the motor disability becomes so severe that treatment aimed at either replenishing DA stores in the brain (e.g., levodopa) or stimulating DA receptors (e.g., DA agonists), or both, is required to alleviate symptoms. Unfortunately, the chronic administration of levodopa often causes motor and psychiatric side effects that may be as debilitating as the disease itself (5). Furthermore, there is no supportive evidence that levodopa therapy impedes the progressive death of SNpc DA neurons; on the contrary, there are speculations that levodopa may contribute to the progressive nature of the disease. Although the actual cause and mechanism of neurodegeneration in PD remains uncertain, it has been hypothesized that the finely tuned balance between the production and the destruction of ROS is upset by either increased ROS formation, decreased ROS detoxification, or both, leading to SNpc DA neuronal death. Over the years, a huge number of factors have been proposed for mediating the speculated ROS attack on SNpc DA neurons. It is our opinion, however, that only a handful of these are credible and warrant in-depth discussion (Table 1).

III. IS DOPAMINE THE CULPRIT?

For a long time, DA was thought of as a "Jekyll and Hyde" agent. On the one hand, DA is the necessary chemical of catecholamine neurotransmission; hence

Table 1 Presumed Contributing Factors in Oxidative Stress in PD

Factors that can stimulate ROS formation
Dopamine metabolism
Autooxidation (nonenzymatic)
Oxidative deamination (enzymatic; MAO)
Neuromelanin
Increased iron content
Impaired mitochondrial electron transport chain activity
Factors that can reduce ROS detoxification
Decrease in activity of ROS scavenging enzymes
Low glutathione peroxidase
Low catalase
Decrease in ROS-scavenging small molecules
Low reduced glutathione
Low ubiquinone

it plays a critical role in proper motor control and other essential neurological functions. On the other hand, as it can engage in ROS-producing biological reactions, DA can be cytotoxic (6). Thus, it may well be that DA neurons are the initiators of their own demise.

A. How Does Dopamine Stimulate ROS Formation?

To date, two mechanisms have been postulated to underlie DA stimulation of ROS (Fig. 1). First of all, because of its catechol moiety, DA is prone to autooxidation in aqueous medium and at physiological pH. DA and other catecholamines undergo a nonenzymatic degradation to ROS as well as to semiquinone and quinone intermediates (Fig. 2) (7). Although the autooxidation of DA occurs readily in vitro (and likely in vivo), its actual cytotoxic role in PD remains unclear. Most arguments supporting the deleterious effects of DA autooxidation derive not from experiments using DA itself but from experiments using its precursor levodopa and the neurotoxin 6-hydroxydopamine—a compound suggested as an endogenous toxin although to date it has never been recovered from PD brains. Second, DA is oxidatively deaminated in the SNpc and in the striatum by the enzyme monoamine oxidase (MAO) which is located in the outer mitochondrial membrane (Fig. 2) (8). This reaction results in a two-electron reduction of oxygen and the production not only of hydrogen peroxide but of superoxide and hydroxyl radicals (7). Of note, while there is some controversy as to whether hydrogen

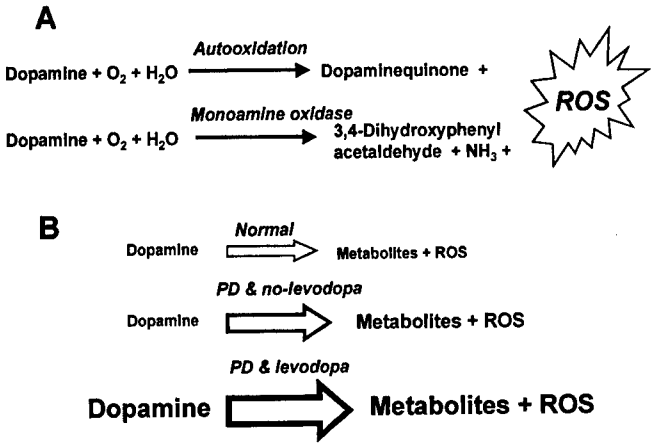

Figure 1 Dopamine metabolism and ROS production. (A) The nonenzymatic and the enzymatic metabolic routes by which dopamine produces ROS. (B) ROS formation is increased in PD due to the augmented dopamine turnover in spared dopamine neurons in the absence or presence of levodopa.

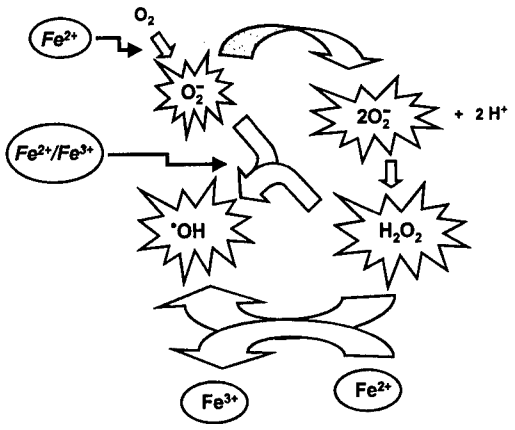

Figure 2 Ferrous ion (Fe^{2+}) can combine with oxygen to produce superoxide radical ($O_2^{\cdot-}$) which, in the presence of hydrogen (H^+), can produce hydrogen peroxide (H_2O_2). Both can react in the presence of Fe^{2+}/ferric (Fe^{3+}) redox couple to produce the hydroxyl radical ($^{\cdot}OH$); this reaction is called the iron-catalyzed Haber-Weiss reaction. Hydroxyl radical can also be produced by the reaction of H_2O_2 with Fe^{2+}; this reaction is called the Fenton reaction. Note the cyclic nature of the iron involvement in ROS production.

peroxide and superoxide radicals can exert direct cytotoxic effects, there is no doubt that the hydroxyl radical is a tissue-damaging reactive species (1).

B. Dopamine and Neurodegeneration

In light of the above, it has been proposed that both the nonenzymatic and enzymatic metabolism of DA produce an ROS burden on the cell which, if not properly detoxified, may become a key factor in the degeneration of DA neurons in PD (6). Relevant to this view is the demonstration that the remaining DA neurons in PD brains are hyperactive and exhibit increased DA turnover (3,9), which presumably increases ROS formation (Fig. 1B). When levodopa is given, these remaining DA neurons are flooded with DA, which increases ROS formation even further (Fig. 1B). This has led to the contention that levodopa therapy and the consequent formation of "excess" DA may promote the progression of PD by increasing the cellular load of ROS. In cell culture, it has been clearly demonstrated that levodopa produces a ROS-related, dose-dependent cytotoxicity (10–12). Also, chronic administration of levodopa enhances DA neuron degeneration in animals with partial lesions of the nigrostriatal DA system (13), but not in animals with intact brain DA systems (14). This observation suggests that levodopa contributes to the further degeneration of an already damaged DA pathway, likely via a ROS-related mechanism. Furthermore, exposure of brain mitochondria to DA causes a significant ROS-mediated inhibition of complex I activity of the electron transport chain (15) associated with a sharp increase in the mitochondrial consumption of the antioxidant glutathione (16,17). These findings are consistent with the view that the metabolism of DA by MAO, a mitochondrial enzyme, can alter mitochondrial function, which in turn may enhance ROS-mediated injury to the cell.

IV. NEUROMELANIN

Neuromelanin is a black pigment found in certain monoaminergic neuron subpopulations of some animal species, including primates (18). This insoluble pigment results from the autooxidation, condensation, and polymerization of DA and its oxidation products (8). Although some neuromelanin accumulates in DA cell bodies during the normal aging process, those SNpc DA neurons that contain the greatest amounts of neuromelanin die preferentially in PD compared to those with less neuromelanin (19). Further, mammalian species that have neuromelanin, such as monkeys, are more sensitive to the deleterious effects of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) than those who do not have neuromelanin, such as mice. All together, these observations suggest

that neuromelanin may not be just a benign pigment but rather another component of the SNpc neuron which, like DA, can turn against its own cell.

How is it that neuromelanin can contribute to the death of SNpc neurons? There are at least three possible mechanisms. First, neuromelanin exhibits an impressive capacity to bind anything that is not standing still, including the active metabolite of MPTP, 4-phenylpyridinium ion (MPP^+) (20), and iron (21), making neuromelanin a potential toxic reservoir within the neuron. For instance, by binding iron, neuromelanin "neutralizes" iron, preventing its participation in ROS production. However, if neuromelanin releases iron in response to cellular events (e.g., oxidative attack on neuromelanin or increased reduced glutathione levels; L. Zecca, personal communication), neuromelanin can become an accomplice in iron-catalyzed ROS production (Fig. 2) (22). Second, neuromelanin also contains redox partners involving quinone, hydroquinone, and semiquinone intermediates (22). These redox partners, under biological conditions, can be responsible for catalyzing the formation of ROS such as superoxide radicals and hydrogen peroxide (22). Third, several breakdown products of neuromelanin are easily oxidized and can exert cytotoxic effects (23). In light of these facts, it is highly plausible that neuromelanin may indeed behave as a ferocious prooxidant in pathological situations such as in PD.

V. METALS AND OXIDATIVE STRESS

Formation of highly reactive tissue-damaging ROS is catalyzed by transition metals such as iron (Fig. 2). In the absence of these metals, it is likely that cells will produce only poorly reactive oxygen species with little potential to cause oxidative damage. Because distribution of transition metals in organs like the brain exhibit striking regional differences, it follows that those brain regions with the highest iron content ought to be at the greatest risk for aggressive oxidative attack. It is thus tempting to implicate iron in SNpc DA neuron degeneration not only because this region of the brain contains a high amount of iron in the normal situation but also because iron content is even higher in PD brains (24–27). In support of this supposition is a recent study showing that increased iron in PD occurs solely in the melanized neurons of the SNpc (28). This result suggests that iron metabolism is specifically altered in SNpc DA neurons in PD, leading to an abnormal accumulation of iron in these neurons. This is plausible because lactoferrin receptors, which facilitate iron entry into cells, are increased most significantly in the remaining melanized neurons in the most damaged area of the SNpc in PD brains (29). However, despite the fact that intracellular iron content is increased in PD brains, one should remember that iron is bound to ferritin as well as to neuromelanin, thus forming a nonreactive complex. The status of ferritin in PD is controversial in that ferritin levels have been reported to

be increased (30) and decreased (27) in PD brains. Therefore, whether increased intracellular iron corresponds, at least in part, to an increase in free iron, which in turn can catalyze oxidative reactions, remains uncertain.

VI. ALTERATIONS IN THE MITOCHONDRIAL ELECTRON TRANSPORT CHAIN

Mitochondria control oxidation–reduction reactions in the cell, generate energy in the form of ATP, and are the main cellular source of ROS through the respiratory chain/oxidative phosphorylation system (1,31). It is believed that mitochondrial function is impaired in postmortem PD midbrains due to a deficit in complex I activity (32,33). Consequently, neurons with lower mitochondrial electron transport chain activity may be subjected to both oxidative stress and energy failure (31). Because this deficit occurs in end-stage PD, a legitimate question is whether the decrease in complex I activity is the cause or the consequence of the neurodegenerative process. To date, there is no definite answer to this important issue. However, studies in animals treated with MPTP suggest that decreases in complex I activity belong on the list of causes rather than consequences, as inhibition of complex I by MPP^+ (which increases ROS formation and decreases ATP production) precedes cell death (34). Since most PD patients receive some chronic anti-PD treatment, it is worth mentioning that high amounts of levodopa and DA can affect brain mitochondrial complex I activity by a ROS-mediated mechanism (15,17,35). These observations suggest that SNpc DA neurons may be subjected to a higher magnitude of chronic oxidative stress originating from the defective mitochondria and, again, could be exacerbated by chronic levodopa therapy (Fig. 1).

VII. ANTIOXIDANT DEFENSE SYSTEMS IN THE BRAIN

In the above sections, we have reviewed several cellular factors about SNpc DA neurons that can underlie the production of ROS and which, by virtue of pathological changes, can participate directly or indirectly in subjecting SNpc DA neurons to oxidative stress. Defense mechanisms exist that limit the levels and the role of ROS in inflicting damage on cellular components (1), as illustrated in Fig. 3. Therefore, while it is unquestionable that a number of factors may contribute to increasing ROS production in SNpc DA neurons, their role must be placed in the context of the natural antioxidant protective arsenal. This suggests that oxidative damage can only be incriminated in PD pathogenesis if the rate of ROS production exceeds that of ROS scavenging. Thus, regardless of the magnitude of ROS production in PD, one may wonder whether there is any evidence supporting the

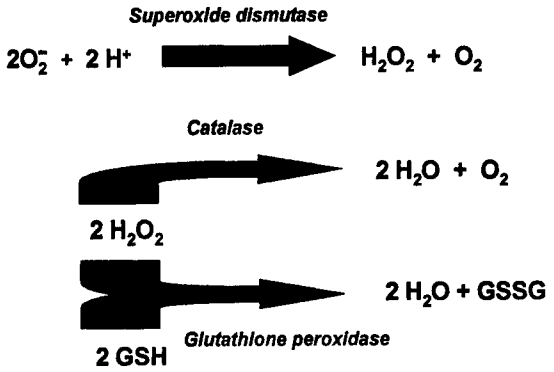

Figure 3 Illustration of ROS-scavenging enzymes.

concomitant weakening of ROS protective mechanisms. The main ROS scavengers are superoxide dismutase (SOD), catalase, and glutathione peroxidase (Fig. 3), as well as several small molecules with strong antioxidant properties, such as glutathione, vitamin C, vitamin E, and ubiquinone.

A. ROS-Scavenging Enzymes

Among the ROS-scavenging enzymes in PD, SOD has received the lion's share of investigation. One finding that makes SOD, and more particularly the cytosolic form of this enzyme, i.e., copper/zinc-SOD (SOD1), appealing in relation to PD is the fact that extremely high amounts of SOD1 are present in SNpc DA neurons (36,37). However, analyses of SOD levels in PD brains show no changes in SOD1 activity in either striatum or SNpc, whereas that of mitochondrial manganese-SOD (SOD2) is significantly increased in both regions (38,39). As opposed to SOD1, which is expressed constitutively, SOD2 is highly inducible in response to an excess of ROS (1). Thus, because it is likely that increased SOD2 activity is protective rather than deleterious, the observed increases in SOD2 in PD strongly suggest that the mitochondrial compartment in PD is subjected to an oxidative stress which, in turn, stimulates the expression of SOD2. Consistent with the neuroprotective effects of increased SOD activity are the observations that a three- to fourfold increase in SOD1 activity promotes DA neuronal survival and nerve fiber sprouting in vitro (37) and stimulates embryonic midbrain graft development in transplanted PD rat models (40). In addition, transgenic mice that overexpress SOD1 are more resistant to MPTP, which stimulates the production of ROS (41). Collectively, these findings support the idea that increased SOD

activity in PD is more likely to be the reflection of a neuroprotective response than part of a destructive phenomenon.

In contrast to SOD, both catalase and glutathione peroxidase activities are reduced in PD brains (42,43). It is surprising, however, that glutathione peroxidase in PD brains is found essentially in SNpc glial cells and not in SNpc neurons (44). This suggests that a deficit in the ROS scavenging system in glial cells, by impairing glial/neuronal cooperation, may trigger neuronal injury, a possibility that would challenge our common "neuronal centrist" view of neurodegeneration. It is also important to note that the reported changes in catalase and glutathione peroxidase activities in PD brains are of small magnitude, implying that they would be insufficient in causing damage in the absence of a concomitant overproduction of ROS.

B. ROS-Scavenging Small Molecules

It is commonly recommended that PD patients take dietary supplements such as vitamins E and C for neuroprotective purposes. Although this recommendation is harmless, its usefulness in PD is quite equivocal, as high intakes of both vitamins do not appear either to lower the risk of developing PD or to slow down its progression (45–48). Furthermore, there is also no indication that levels of vitamin E are abnormal in PD brains (49). In contrast, both ubiquinone (50) and glutathione (51) levels appear abnormally low in PD, which may be of pathological significance, since both compounds play an important antioxidant role in the brain and are present in high amounts within mitochondria. Of interest are the observations that ubiquinone supplements are well tolerated and seem to increase complex I activity in PD patients (52), as well as to attenuate MPTP toxicity in mice (53). No information seems to be available regarding the use and potential benefit of glutathione or its precursor, *N*-acetylcysteine, in PD.

VIII. ROS-INDUCED NEURONAL DAMAGE IN THE SUBSTANTIA NIGRA PARS COMPACTA

There is little doubt that neurons (or any other cell types for that matter) would die if subjected to the postulated harsh oxidative insults likely to be encountered in certain acute neurological disorders such as ischemia and stroke. In PD, however, there is no evidence that SNpc DA neurons face such a severe insult. On the other hand, because PD is a chronic disorder, a much milder oxidative stress may cause a buildup of ROS-mediated damage over several years, leading to progressive cellular dysfunction which ultimately commits the neuron to die.

Furthermore, it is not even clear if SNpc DA neurons can actually die following oxidative stress. This problem can be addressed in experimental models

of PD, in which the moment of the injury is known and the neurodegenerative process can be followed from beginning to end (54,55). The two most popular and extensively validated models of PD are those produced by the neurotoxins 6-hydroxydopamine and MPTP. Both compounds stimulate ROS production, albeit by quite different and distinct mechanisms.

6-Hydroxydopamine is believed to kill cells (more specifically, catecholaminergic neurons) by the production of hydrogen peroxide, superoxide, and hydroxyl radicals following its autooxidation (56,57). The specificity of action depends on its uptake and accumulation into catecholaminergic neurons and terminals (58–60). Since 6-hydroxydopamine does not cross the blood–brain barrier, its systemic administration only affects the catecholaminergic structures of the peripheral nervous system. On the other hand, when 6-hydroxydopamine is injected locally into the striatum, the median forebrain bundle, or the SNpc, it selectively destroys the nigrostriatal DA pathway (58–60). Because of this, 6-hydroxydopamine is often used to produce a rat model of PD. Strengthening the role of ROS in the 6-hydroxydopamine-induced neurotoxicity is the fact that various antioxidants attenuate its deleterious effects (61,62).

As opposed to 6-hydroxydopamine, MPTP does cross the blood–brain barrier, and systemic administration damages DA neurons and reproduces most of the clinical and pathological hallmarks of PD (63). Its metabolism is a complex multistep process that can stimulate ROS production by both mitochondrial (64–66) and cytosolic (67,68) mechanisms. In addition, MPTP's mode of action involves not only ROS such as superoxide anion and hydroxyl radicals (64,69,70), but also nitric oxide (71). Here again, strategies that either increase the protection against ROS or hamper the production of ROS attenuate MPTP-induced toxicity (41,70,71). Thus, both models provide compelling evidence that oxidative stress unequivocally kills SNpc DA neurons. The specificity of the lesions caused by both 6-hydroxydopamine and MPTP, however, result from the specific uptake of these neurotoxins into the DA neurons and do not speak to the specific susceptibility of DA neurons to oxidative stress over any other subpopulation of neurons.

Other informative models have been used over the years to test whether the different factors presented in Table 1 can actually ignite ROS attack on SNpc DA neurons. For instance, it was shown that stereotaxic injection of synthetic neuromelanin into rodent brains did not cause cell death by itself but potentiated MPTP-induced neurotoxicity (72). One caveat with this study is that the injected synthetic neuromelanin most likely remained in the extracellular space and did not enter the neurons. Thus, based on this work, we cannot exclude neuromelanin as a neurotoxic compound. More importantly, the stereotaxic injection of MPP^+ and rotenone, two mitochondrial poisons, produced severe nigrostriatal damage (73), illustrating the importance of mitochondrial impairment in the death of SNpc DA neurons. As critical as ROS-scavenging enzymes appear to be in the detoxification of ROS, it appears that ablation of extracellular and cytosolic SOD or glutathione peroxidase in knockout mice had negligible effects on the develop-

ment and survival of SNpc DA neurons (74–76). On the other hand, increased activity of ROS scavenging enzymes, and particularly of cytosolic SOD, promoted the development and survival of SNpc DA neurons and rendered SNpc DA neurons more resistant to oxidative stress (37,41).

IX. TARGETS OF ROS

There is no doubt that by combining the various potentially deleterious factors described above one can produce an explosive scenario that could convince even the most die-hard skeptics that SNpc DA neurons are, or can be, the sites of oxidative attack. However, one has to keep in mind that all of these findings, appealing as they may be, are strictly circumstantial. Indeed, SNpc DA neurons do die, and clear abnormalities in both the production of ROS and in the detoxification of ROS are observed in SNpc, but whether the former is due to the latter has yet to be unequivocally demonstrated. While the experimental models strongly support this contention, none of the postmortem studies have been able to settle the issue.

ROS can attack virtually all cellular components, including nucleic acids, proteins, and lipids (1). Some of the ROS-mediated alterations in cellular components are stable modifications and can readily be quantified. Therefore, the demonstration that cellular elements critical to cellular function, survival, or both are damaged by ROS may provide invaluable support for the role of oxidative stress in the pathogenesis of PD. It should also be pointed out that while ROS can theoretically affect all cellular elements, in reality, it is frequently specific elements that are preferentially damaged. Consequently, it is the general consensus that an accurate demonstration of the existence and the severity of ROS-mediated damage can only be achieved by examining more than a single marker of ROS-mediated damage, and probably by using more than one technique.

The brain is extremely rich in phospholipids and polyunsaturated free fatty acids (PUFAs), both of which are highly susceptible to ROS attack. Following ROS-mediated damage of phospholipids and PUFAs, plasma membrane and intracellular organelles, whose structure and function rely on a normal protein/lipid bilayer organization, can be dangerously jeopardized. In PD, the concentration of PUFAs in the nigra is decreased, while that of malondialdehyde, a marker of lipid oxidation, is increased (77). Additional evidence of lipid oxidation in PD is provided by the demonstration that 4-hydroxy-2-nonenal, a lipophilic product of the peroxidation of membrane-bound arachidonic acid that accumulates in membranes, is increased about fourfold in the spinal fluid of PD patients (78). Levels of markers of oxidative damage to proteins, such as carbonyl modifications of soluble proteins, are also significantly increased in postmortem samples of substantia nigra in PD brains as compared to controls (79). DNA also does not escape ROS attack; for example, deoxyguanosine is converted to 8-hydroxydeoxyguanosine (8-OHdG) (80). Like

the other markers of oxidative damage, 8-OHdG is also markedly increased in postmortem samples of substantia nigra in PD brains (81).

X. CONCLUSION

PD is a slow, progressive neurodegenerative disorder. The evidence presented here views ROS as instrumental in fostering oxidative stress, leading to the eventual destruction of SNpc DA neurons, which are the primary targets in PD. The slow, progressive nature of PD suggests that ongoing mild increases in oxidative stress, rather than a one-time dramatic insult, has the potential to be pathogenic. We remain unable, however, to point to a single abnormality in ROS metabolism that we can confidently convict as the executioner of SNpc DA neurons. On the other hand, if we try to reconcile all of the different experimental evidence presented above, we could raise the possibility of a conspiracy theory in which SNpc DA neuronal death is mediated by a group of otherwise unimpressive attackers that combine their forces to produce SNpc DA neurodegeneration. In most instances, we are used to thinking in terms of "one disease–one mechanism," making the idea of a conspiracy theory difficult to envision in the case of PD. But if one looks at PD as the outcome of a cascade of deleterious events, even if some of the observed abnormalities are secondary, albeit meaningful, the conspiracy theory becomes much more plausible.

In conclusion, we believe that compelling evidence now exists to support the role of ROS in the pathogenesis of PD. We also believe that more mechanistic studies are still needed to clarify the actual importance of certain alterations reported in the literature, and to distinguish those agents that cause real deleterious effects from those that are unlikely to have any real pathological role in the development and progression of PD.

ABBREVIATIONS

DA	dopamine
MAO	monoamine oxidase
MPP^+	1-methyl-4-phenylpyridinium ion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
PD	Parkinson's disease
PUFA	polyunsaturated free fatty acid
ROS	reactive oxygen species
SNpc	substantia nigra pars compacta
SOD	superoxide dismutase
SOD1	copper/zinc-SOD
SOD2	manganese-SOD
8-OHdG	8-hydroxydeoxyguanosine

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RAPID COMMUNICATION

Oxidative post-translational modifications of α-synuclein in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease

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Abstract

Structural and functional alterations of α-synuclein is a presumed culprit in the demise of dopaminergic neurons in Parkinson's disease (PD). α-Synuclein mutations are found in familial but not in sporadic PD, raising the hypothesis that effects similar to those of familial PD-linked α-synuclein mutations may be achieved by oxidative post-translational modifications. Here, we show that wild-type α-synuclein is a selective target for nitration following peroxynitrite exposure of stably transfected HEK293 cells. Nitration of α-synuclein also occurs in the mouse striatum and ventral midbrain following administration of the parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Conversely, β-synuclein and synaptophysin were not nitrated in MPTP-intoxicated mice. Our data demonstrate that α-synuclein is a target for tyrosine nitration, which, by disrupting its biophysical properties, may be relevant to the putative role of α-synuclein in the neurodegeneration associated with MPTP toxicity and with PD.

Keywords: dopaminergic neurons, MPTP, neurodegeneration, Parkinson's disease, substantia nigra, synuclein.
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Parkinson's disease (PD) is a common neurodegenerative disorder that can be of either familial or non-familial (i.e. sporadic) etiology (Fahn and Przedborski 2000). Cardinal clinical features of PD include tremor, stiffness and slowness of movement, all of which are attributed to the dramatic loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Fahn and Przedborski 2000). Mutations in the presynaptic protein α-synuclein (α-syn) are associated with a familial form of PD (Polymeropoulos *et al.* 1997) that is clinically and pathologically indistinguishable from the most common sporadic form of this disabling neurodegenerative disorder. Mutant α-syn cytotoxicity is likely related to the fact that both of the identified point mutations may enhance the propensity of α-syn to interact with other intracellular proteins and increase its tendency to aggregate (Conway *et al.* 1998; El-Agnaf *et al.* 1998; Engelender *et al.* 1999; Giasson *et al.* 1999; Narhi *et al.* 1999). Although similar α-syn mutations are not found in sporadic PD (Golbe 1999), mounting evidence indicates that α-syn may also play a deleterious role in sporadic PD (Spillantini *et al.* 1997, 1998). Relevant to these observations, we have recently reported widespread nitration of proteins, which is a consequence of the reaction of nitrating agents such as peroxynitrite with proteins (Ischiropoulos and al Mehdi 1995), in Lewy bodies (LBs) in sporadic PD, in dementia with LBs (DLB) and in the LB variant of Alzheimer's disease (Duda *et al.* 2000a). Moreover, using specific monoclonal antibodies that recognize only nitrated α-syn, we have also demonstrated that α-syn is the major protein that is modified by nitration in the LBs of the above neurodegenerative disorders as well as in neuronal and glial cytoplasmic inclusions (GCIs) in multiple system atrophy (MSA) and in Hallervorden–Spatz disease (HSD) (Giasson *et al.* 2000a). These data provide, for the first time, compelling evidence for the presence of nitrative stress and formation of nitrating agents in human neurodegenerative disorders. They also substantiate the hypothesis that oxidative stress is a leading pathogenic mechanism in neurodegenerative disorders including PD (Przedborski and Jackson-Lewis 2000). In addition, exposure of wild-type human α-syn to nitrating agents *in vitro*

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Abbreviations used: α-syn, α-synuclein; β-syn, β-synuclein; LBs, Lewy bodies; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 3-NT, 3-nitrotyrosine; PD, Parkinson's disease; SNpc, substantia nigra pars compacta.

causes nitration and cross-linking via the formation of dityrosine, recapitulating some aspects of the α-syn extracted from the brains of humans with α-syn aggregates (Duda *et al.* 2000b; Galvin *et al.* 2000; Giasson *et al.* 2000a). Extending these observations and consistent with the oxidative-nitrative stress hypothesis, we now present compelling evidence that α-syn is a specific target for tyrosine nitration in stably transfected cells challenged with peroxynitrite, and in mice intoxicated with the parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tertahydropyridine (MPTP) (Przedborski *et al.* 2000a).

Experimental procedures

Stable transfection of HEK 293 cells

Syn plasmids were constructed by inserting human α- or β-syn cDNAs (Jakes *et al.* 1994) into the mammalian expression vector pcDNA 3.1+ (Invitrogen, Carlsbad, CA, USA). HEK293 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in 90% Dulbecco's modified Eagle's medium (high glucose), 10% fetal bovine serum, 2 mM L-glutamine and antibiotics. Cells were transfected with α-syn/pcDNA3.1+ or β-syn/pcDNA3.1+ using calcium phosphate precipitation buffered with N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES) (Chen and Okayama 1997). One day following transfection, the cells were re-plated on 10-cm dishes and selection with Geneticin (500 μg/mL) (Life Technologies, Rockville, MD, USA) was initiated 24 h later. Individual stable clones were isolated with glass cylinders and detached from the dish with trypsin. Stable clones were re-plated and maintained in culture medium with Geneticin. Clones expressing high levels of syn were screened by western blot analysis.

Exposure of cells to peroxynitrite

Cells re-plated on six-well dishes in fresh culture medium were exposed to peroxynitrite by the addition of small drops above the cell surface and followed by rapid mixing to give a final concentration of 1 mM. The concentration of peroxynitrite was measured by the increase in absorbance at 302 nm in 1.2 M NaOH as described previously (Ara *et al.* 1998). The pH of the buffer was measured at the end of the exposure and was the same as before each addition of peroxynitrite. For all experiments, at the end of the incubation period, cells were washed with Earle's balanced salt solution, scraped-off of the plates and centrifuged at 8000 *g* for 5 min. The pellet was solubilized with lysis buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 4 mM EGTA, 10% glycerol, 1% Triton-X 100]. As a control, peroxynitrite was allowed to decompose in cell media prior to exposure to the cells. This experiment controlled for hydrogen peroxide and nitrite, which are found in peroxynitrite solutions.

Mice and MPTP administration

Eight-week-old male C57/bl mice (22–25 g, Charles River Breeding Laboratories, Wilmington, MA, USA) were used. Animals were housed with three per cage in a temperature-controlled room under a 12-h light/12-h dark cycle with free access to food and water. Mice used in this study were treated according to the NIH guidelines for *Care and Use of Laboratory Animals* and with the approval of Columbia University's Institutional Animal Care and Use Committee. On the day of the experiment, mice received four i.p. injections of MPTP-HCl (20 mg/kg) in saline at 2 h intervals and were killed at 4 and 24 h post-injection; control mice received saline injections only. MPTP use and safety precautions were as described previously (Przedborski *et al.* 2000b). Frozen mouse striatal and ventral midbrain samples were homogenized (Polytron) in 5.0 mL of extraction buffer (phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride and aprotinin at 10 mg/mL) at 4°C.

Immunoprecipitation and western blotting

Solubilized extracts from cells and mice were briefly sonicated and centrifuged (325 *g* for 5 min) to remove cellular debris. Protein concentration was determined using a BCA kit (Pierce, Rockford, IL, USA). Solubilized proteins (2 mg) were precleared (45 min, 4°C) with 15 μL of Gamma bind plus Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and the supernatant was incubated (16 h, 4°C) with a rabbit anti-3-nitrotyrosine (3-NT) polyclonal antibody previously characterized (Beckman *et al.* 1994; Ye *et al.* 1996), or a mouse anti-α-syn monoclonal antibody (Syn-1) (Transduction Laboratories, Lexington, KY, USA). Immune complexes were absorbed (1.5 h, 4°C) to 50 μL of Gamma Bind plus Sepharose, extensively washed with extraction buffer by sequential sedimentation and resuspension, eluted in sample loading buffer by heating to 95°C for 5 min, and resolved by SDS–PAGE on 12–15% polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose or PVDF membranes, which were blocked with 5% non-fat dry milk in 1 × TBS, 0.1% Tween 20 for 1 h. Incubation with one of the primary antibodies was performed overnight at 4°C using either Syn-1, anti-β-syn (a gift from Dr S. Nakajo, Tokyo, Japan), Syn 207 (Giasson *et al.* 2000b), anti-synaptophysin (a gift from Dr Honer, Albert Einstein College of Medicine, NY, USA) or anti-3-NT antibody. Incubation with a secondary anti-mouse- or anti-rabbit-conjugated horseradish peroxidase antibody was performed at 25°C for 1 h. After washing in 1 × TBS, 0.1% Tween-20, blots were developed with Super Signal Ultra chemiluminiscence (Pierce) and exposed to Kodak BetaMax film.

Results and discussion

In the present study, we demonstrate that α-syn is a specific target for tyrosine nitration in a cell model as well as in the mouse brain after MPTP intoxication. First, non-transfected HEK293 cells and HEK293 cells overexpressing human α-syn or β-syn were exposed to

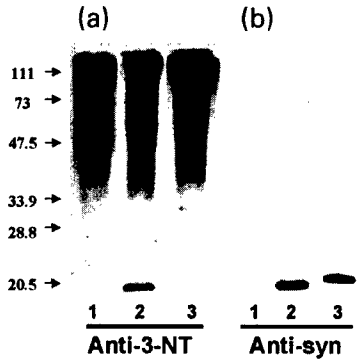

Fig. 1 Exposure of HEK293 cells to peroxynitrite results in the nitration of cellular proteins. All of the cells were exposed, under the same conditions, to 1 mM chemically synthesized peroxynitrite. (a) Western blot analysis using an anti-NT polyclonal antibody: lane 1 – non-transfected cells; lane 2 – cells expressing human α-syn; lane 3 – cells expressing β-syn. (b) The same as (a) but developed using an anti-α-syn antibody (lane 1 and 2) or using the antibody Syn 207, which specifically recognizes β-syn. Fifty μg of total protein was loaded onto each lane of the polyacrylamide gel. The molecular masses of markers are indicated to the left of the blots.

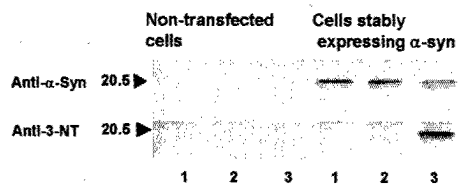

Fig. 2 Immunoprecipitation of α-syn followed by western blotting with antibodies to α-syn or NT reveals nitration of the protein after the exposure of HEK293 cells to peroxynitrite. Cells were untreated (lanes 1), or exposed to decomposed (lanes 2) or fresh (lanes 3) peroxynitrite.

peroxynitrite, an oxidizing and nitrating agent (Ischiropoulos and al Mehdi 1995). This treatment resulted in the nitration of a number of proteins as demonstrated by the western blot analysis using an anti-NT antibody (Fig. 1a). However, only HEK293 cells transfected with α-syn showed a nitrated protein band with the molecular mass corresponding to α-syn. To demonstrate that α-syn was indeed nitrated, the total protein extract was subjected to immunoprecipitation using an anti-α-syn antibody and then the recovered immunoprecipitated protein was probed with the anti-3-NT antibody. This experiment confirmed that a significant fraction of the immunoprecipitated α-syn was definitely nitrated in the cells exposed to peroxynitrite (Fig. 2, lane 3), but not in untreated cells or in cells exposed to decomposed reagent (Fig. 2, lanes 1 and 2).

Given these results, we then proceeded to assess whether α-syn was nitrated in the MPTP mouse model of PD. The use of this particular experimental model has been motivated by the fact that, thus far, significant insights into the pathogenesis of PD have been achieved using this neurotoxin, which replicates in humans and in non-human primates a severe and irreversible PD-like syndrome, with concomitant degeneration of dopaminergic neurons (Przedborski *et al.* 2000a). Moreover, several studies have indicated that reactive nitrogen species and tyrosine nitration not only occur in this model but also participate in the MPTP neurotoxic process (Schulz *et al.* 1995; Przedborski *et al.* 1996; Ara *et al.* 1998; Mandir *et al.* 1999; Pennathur *et al.* 1999).

Immunoprecipitation of α-syn was performed as above from striatum and ventral midbrain, the two main targets of MPTP neurotoxicity (Przedborski *et al.* 2000a). Immunoprecipitated α-syn from striatum and ventral midbrain was selectively nitrated 4 h after the MPTP administration (Fig. 3). Conversely, immunoprecipitation of α-syn from striatum and ventral midbrain of saline-injected mice, also at 4 h post-injection, did not reveal any detectable nitration of the protein (Fig. 3). In contrast to the robust tyrosine nitration of α-syn (Figs 3a and b), no tyrosine nitration was detected in two other presynaptic proteins, β-syn and synaptophysin, following a similar MPTP administration (Figs 3c and d). This observation is consistent with our previous finding that only selected proteins are tyrosine-nitrated after MPTP exposure (Ara *et al.* 1998; Ischiropoulos 1998) and with the observation that α-syn, but not β-syn, is nitrated after exposure of cells to the same peroxynitrite challenge. Moreover, this observation is consistent with the demonstrations that nitrated α-syn is present in the hallmark lesions in a number of human neurodegenerative synucleinopathies (Duda *et al.* 2000a; Giasson *et al.* 2000a).

The higher efficiency of α-syn nitration is likely to be caused by the unstructured conformation of the protein in aqueous solution, which exposes all four tyrosine residues to the solvent phase and increases the probability of the reaction with nitrating agents. Moreover, glutamate residues, a structural conformation associated with enhanced susceptibility of tyrosine to nitration, are near all three tyrosine residues (125, 133 and 136) in the carboxy terminal domain of α-syn. Indeed, purified human α-syn exposed to the nitrating agent *in vitro* and analyzed by

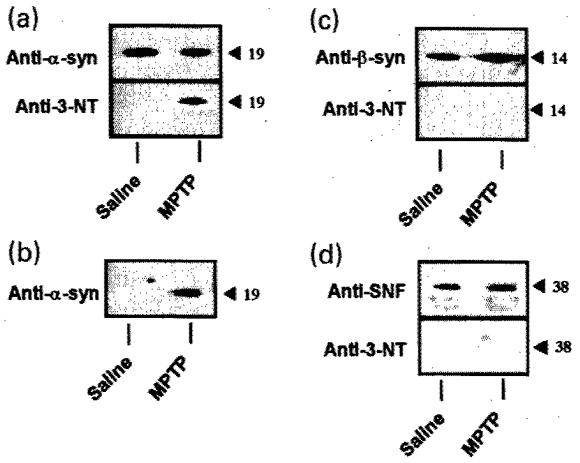

Fig. 3 Tyrosine nitration of striatal α-syn, but not of β-syn or synaptophysin, following MPTP injection to mice. Following administration of MPTP (4 h after the last injection) or vehicle (saline), striatal proteins were immunoprecipitated (IP) using: anti-α-syn (a); anti-NT (b); anti-β-syn (c); or anti-synaptophysin (d) as described in Experimental procedures. After SDS–PAGE and transfer of proteins onto nitrocellulose membrane, immunoblots were probed with anti-NT (lower panels of a, c and d), anti-α-syn (a – top panel and b), anti-β-syn (c – top panel) and anti-synaptophysin (d – top panel) as described in Experimental procedures.

electrospray mass spectrometry, revealed that nitration of α-syn occurs at all four tyrosine residues (Souza *et al.* 2000). In addition to nitration, exposure of α-syn to nitrating agents also results in the stable cross-linking of the protein via the formation of dityrosine (Souza *et al.* 2000). In contrast, β-syn is nitrated to a lesser extent than α-syn *in vitro* and does not form stable *O-O'*dityrosine crosslinks after exposure to nitrating agents, despite the presence of all four conserved tyrosine residues in both proteins (Souza *et al.* 2000). More significantly, we show here that in two *in vivo* models, α-syn is selectively nitrated, whereas nitration of β-syn is below detectable limits. The preferential nitration and oxidation of tyrosine residues in α-syn could be caused by the accessibility of tyrosine residues to nitrating agents and by the presence of the protein in close proximity to the site(s) of generation of the nitrating agent. Our results raise the possibility that both syn proteins may have different confirmations, or that β-syn may be protected from oxidation, perhaps by different interacting partners *in vivo*. We have previously argued that proximity to sites of superoxide generation may be important in determining proteins modified by nitration, as overexpression of superoxide dismutase and superoxide mimetics have been shown to prevent the nitration of proteins *in vivo* and in cell models (Ara *et al.* 1998; Cuzzocrea *et al.* 2000; Pong *et al.* 2000).

The significance of the tyrosine nitration of α-syn remains unclear. Tyrosine nitration induces secondary and tertiary structural alterations, which may critically modify protein functions (Ischiropoulos 1998). The change in the ionization state of the modified protein induced by a local shift in the pKa from 10.01 of tyrosine to 7.5 of 3-NT, and the consequent changes in hydrophobicity and conformation, may facilitate interactions with other proteins, thereby promoting protein aggregation. Preliminary data indeed indicate an increased adherence of mouse brain extracts to nitrated α-syn compared with the unmodified wild-type protein (Chen *et al.*, unpublished observation).

Collectively, the data indicate that α-syn is a preferential target for oxidative stress-mediated post-translational modifications. These

alterations may trigger abnormal protein compartmentalization and aggregation, two phenomena that are potential culprits for the neurodegeneration process in PD.

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REVIEW ARTICLE

The parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): a technical review of its utility and safety

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Parkinson's disease (PD) is a common disabling neurodegenerative disorder the cardinal clinical features of which include tremor, rigidity and slowness of movement (Fahn and Przedborski 2000). These symptoms are attributed mainly to a profound reduction of dopamine in the striatum due to a dramatic loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Fahn and Przedborski 2000). Thus far, both the cause and the mechanisms of PD remain unknown. Over the years, investigators have used experimental models of PD produced by several compounds such as reserpine, 6-hydroxydopamine, methamphetamine, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to provide insights into the mechanisms responsible for the demise of dopaminergic neurons in PD. To this end, MPTP has emerged unquestionably as a popular tool for inducing a model of PD in a variety of animal species including monkeys, rodents, cats, and pigs (Kopin and Markey 1988). The sensitivity to MPTP and therefore its ability to induce parkinsonism closely follows the phylogenetic tree where the species most closely related to humans are the most vulnerable to this neurotoxin. Due to the significant neurotoxicity of MPTP, it is important that researchers appreciate the potential hazards of this toxin. Given this, the purpose of this review is to inform the researcher of the hazardous nature of MPTP and to provide guidance for its safe handling and use.

MPTP models of PD

MPTP is a by-product of the chemical synthesis of a meperidine analog with potent heroin-like effects. MPTP can induce a parkinsonian syndrome in humans almost indistinguishable from PD (Langston and Irwin 1986). Recognition of MPTP as a neurotoxin occured early in 1982, when several young drug addicts mysteriously developed a profound parkinsonian syndrome after the intravenous use of street preparations of meperidine analogs which, unknown to anyone, were contaminated with MPTP (Langston *et al.* 1983). In humans and non-human primates, depending on the regimen used, MPTP can produce an irreversible and severe parkinsonian syndrome that replicates almost all of the features of PD, including tremor, rigidity, slowness of movement, postural instability, and even freezing; in non-human primates, a resting tremor characteristic of PD has only been demonstrated convincingly in the African green monkey (Tetrud *et al.* 1986). The responses, as well as the complications, to traditional antiparkinsonian therapies are virtually identical to those seen in PD. It is believed that in PD the neurodegenerative process occurs over several years, while the most active phase of neurodegeneration is completed within a few days following MPTP administration (Langston 1987;

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Abbreviations used: MAO-B, monoamine oxidase B; $MPDP^+$, 1-methyl-4-phenyl-2,3-dihydropyridinium; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MSDS, material safety data sheet, PD, Parkinson's disease; PPE, personal protective equipment; SNpc, substantia nigra pars compacta.

Jackson-Lewis *et al.* 1995). However, recent data suggest that, following the main phase of neuronal death, MPTP-induced neurodegeneration may continue to progress 'silently' over several decades, at least in humans intoxicated with MPTP (Vingerhoets *et al.* 1994; Langston *et al.* 1999). Except for four cases (Davis *et al.* 1979; Langston *et al.* 1999), no human pathological material has been available for studies and thus, the comparison between PD and the MPTP model is largely limited to primates (Forno *et al.* 1993). Neuropathological data show that MPTP administration causes damage to the nigrostriatal dopaminergic pathway identical to that seen in PD (Agid *et al.* 1987), yet there is a resemblance that goes beyond the loss of SNpc dopaminergic neurons. Like PD, MPTP causes greater loss of dopaminergic neurons in SNpc than in ventral tegmental area (Seniuk *et al.* 1990; Muthane *et al.* 1994) and, at least in monkeys treated with low doses of MPTP but not in humans, greater degeneration of dopaminergic nerve terminals in the putamen than in the caudate nucleus (Moratalla *et al.* 1992; Snow *et al.* 2000). However, two typical neuropathologic features of PD have, until now, been lacking in the MPTP model. First, except for SNpc, pigmented nuclei such as locus coeruleus have been spared, according to most published reports. Second, the eosinophilic intraneuronal inclusions, called Lewy bodies, so characteristic of PD, thus far, have not been convincingly observed in MPTP-induced parkinsonism (Forno *et al.* 1993), although, in MPTP-injected monkeys, intraneuronal inclusions reminiscent of Lewy bodies have been described (Forno *et al.* 1986).

Modes of administration

To date, the most frequently used animals for MPTP studies are monkeys, mice and rats. The administration of MPTP through a number of different routes using different dosing regimens has led to the development of several distinct models, each characterized by some unique behavioral and/or biochemical features. The manner in which these models were developed is based on the concept of delivering MPTP in a fashion that creates the most severe and stable form of SNpc damage with the least number of undesirable consequences such as acute death, dehydration and malnutrition. Although MPTP can be given by a number of different routes, including gavage and stereotaxic injection into the brain, the most common, reliable, and reproducible lesion is provided by its systemic administration (i.e. subcutaneous, intravenous, intraperitoneal or intramuscular).

Monkeys

The most commonly used regimens in monkeys are the multiple intraperitoneal or intramuscular injections and the intracarotid infusion of MPTP (Petzinger and Langston 1998). The former is easy to perform and produces a bilateral parkinsonian syndrome. However, often the monkey exhibits a generalized parkinsonian syndrome so severe that chronic administration of levodopa is required to enable the animal to eat and drink adequately (Petzinger and Langston 1998). On the other hand, the unilateral intracarotid infusion is technically more difficult, but causes symptoms mainly on one side (Bankiewicz *et al.* 1986; Przedborski *et al.* 1991), which enables the monkey to maintain normal nutrition and hydration without the use levodopa.

For many years monkeys were mainly, if not exclusively, treated with harsh regimens of MPTP to produce an acute and severe dopaminergic neurodegeneration (Petzinger and Langston 1998). More recently, several investigators have treated monkeys with low doses of MPTP (e.g. 0.05 mg/kg 2–3-times per week) for a prolonged period of time (i.e. weeks to months) in an attempt to better model the slow neurodegenerative process of PD (Schneider and Roeltgen 1993; Bezard *et al.* 1997; Schneider *et al.* 1999). While both the acute and the chronic MPTP-monkey models are appropriate for the testing of experimental therapies aimed at alleviating PD symptoms, it is the chronic model that is, presumably, the most suitable for the testing of neuroprotective strategies.

Mice

In addition to monkeys, many other mammalian species are also susceptible to MPTP (Kopin and Markey 1988; Heikkila *et al.* 1989; Przedborski *et al.* 2000). Mice have become the most commonly used species for both technical and financial reasons. However, several problems need to be emphasized. First, mice are much less sensitive to MPTP than monkeys; thus, much higher doses are required to produce significant SNpc damage in this animal species, presenting a far greater hazardous situation. Second, in contrast to the situation in monkeys, mice treated with MPTP do not develop parkinsonism. Third, the magnitude of nigrostriatal damage depends on the dose and dosing schedule (Sonsalla and Heikkila 1986).

Rats

The use of MPTP in rats presents an interesting situation (Kopin and Markey 1988). For instance, rats injected with mg/kg doses of MPTP comparable to those used in mice do not exhibit any significant dopaminergic neurodegeneration (Giovanni *et al.* 1994a; Giovanni *et al.* 1994b). Conversely, rats injected with much higher doses of MPTP do exhibit significant dopaminergic neurodegeneration (Giovanni *et al.* 1994a; Giovanni *et al.* 1994b) although, at these high doses, rats have to be pretreated with guanethidine to prevent dramatic peripheral catecholamine release and extensive mortality (Giovanni *et al.* 1994a). These findings indicate that rats are relatively insensitive to MPTP, but regardless of this drawback, rats continue to be used often in MPTP studies (Storey *et al.* 1992; Giovanni *et al.* 1994a; Giovanni

et al. 1994b; Staal and Sonsalla 2000; Staal *et al.* 2000). In rats, the systemic administration of MPTP is rarely used and the vast majority of studies involve the stereotaxic infusion of MPTP's toxic metabolite, 1-methyl-4-phenylpyridinium (MPP^+) (Storey *et al.* 1992; Giovanni *et al.* 1994a; Giovanni *et al.* 1994b; Staal and Sonsalla 2000; Staal *et al.* 2000).

Intervening factors

Several factors influence the reproducibility of the lesion in monkeys, rats, and mice. However, to our knowledge, the extensive and systematic assessment of these factors has only been done in mice, and can be found in the following references (Heikkila *et al.* 1989; Giovanni *et al.* 1991; Giovanni *et al.* 1994a; Giovanni *et al.* 1994b; Miller *et al.* 1998; Hamre *et al.* 1999; Staal and Sonsalla 2000), the highlights of which can be summarized as follows: different strains of mice (and even within a given strain obtained from different vendors) can exhibit strikingly distinct sensitivity to MPTP. This differential sensitivity acts in an autosomal dominant fashion (Hamre *et al.* 1999). Gender, age, and body weight are also factors that modulate MPTP sensitivity as well as reproducibility of the lesion, in that female mice are less sensitive and exhibit more variability in the extent of damage than males, as do mice younger than 8 weeks and lighter than 25 g. From our experience, optimal reproducibility in MPTP neurotoxicity is obtained using male C57 BL/6 mice 8–10 weeks of age and 25–30 g in weight. Also of importance is that, following MPTP administration, some mice will die within the first 48 h postinjection; note that C57 BL/6 mice from different vendors exhibit dramatically different magnitudes of acute lethality, ranging from 5% to 90%. This common issue is unlikely related to a toxic effect in the central nervous system but rather toxicity to the peripheral nervous and cardiovascular systems. Although, to our knowledge, this possibility has never been formally studied, we believe that, following acute MPTP administration, mice develop fatal alterations in heart rate and blood pressure. Moreover, MPTP intoxication causes a transient drop in body temperature, which not only can modulate the extent of dopaminergic damage (Moy *et al.* 1998), but can also contribute to acute lethality. Death rate can be reduced by maintaining the body temperature of the injected mice using a temperature-controlled warming pad (do not use a lamp, which can kill mice by overheating them as there is no control of the temperature).

Metabolism of MPTP

MPTP has a complex multistep metabolism (Tipton and Singer 1993; Przedborski *et al.* 2000). It is highly lipophilic, and freely and rapidly crosses the blood–brain barrier. Within a minute after MPTP injection, levels of the toxin are detectable in the brain (Markey *et al.* 1984). Once in the brain, MPTP is metabolized to 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) by the enzyme monoamine oxidase B (MAO-B) in non-dopaminergic cells. Then $MPDP^+$ is oxidized to the active MPTP metabolite, MPP^+, which is then released into the extracellular space, where it is taken up by the dopamine transporter and is concentrated within dopaminergic neurons, where it exerts its toxic effects. The essential role of these different metabolic steps in MPTP-induced neurotoxicity and the fact that MPP^+ is the actual culprit are demonstrated by the following observations: (1) pretreatment with MAO-B inhibitors such as deprenyl prevents MPTP biotransformation to MPP^+ and blocks dopaminergic toxicity (Heikkila *et al.* 1984; Markey *et al.* 1984); (2) pretreatment with dopamine uptake inhibitors (e.g. mazindol) prevents MPP^+ entry into dopaminergic neurons and also blocks dopaminergic toxicity (Javitch *et al.* 1985), at least in mice; and (3) striatal MPP^+ content correlates linearly with dopaminergic toxicity in mice (Giovanni *et al.* 1991).

Body distribution and environmental contamination

Knowing where MPTP and its toxic metabolite, MPP^+, accumulate both inside and outside of the body of the injected animal following MPTP administration is germane to the formulation of any set of standard practices for the safe use of MPTP.

Following MPTP administration to both mice and monkeys, only the interior surfaces of the cage, the surfaces that the animals and/or their excreta could physically touch, including food and drinking bottle, are contaminated with MPTP and its metabolites (Yang *et al.* 1988). Conversely, no evidence of contamination is found outside of the cage or on the outside surrounding surfaces (Yang *et al.* 1988). At two days postinjection, 70% of the total injected dose of MPTP and its metabolites is recovered from the inside cage-wash, urine and feces, of which about 15% in mice and 2% in monkeys is unmetabolized MPTP, while the rest is due to MPTP metabolites, such as MPP^+. Moreover, it appears that the excretion of unmetabolized MPTP occurs mainly during the first day postinjection, while mainly MPTP metabolites are excreted up to 3 days postinjection (Yang *et al.* 1988). There is no evidence either in mice or in monkeys that MPTP and its metabolites are still being excreted after 3 days post MPTP administration. Although high concentrations of MPTP are found in the bile, the main route of MPTP excretion is the urine (Johannessen *et al.* 1986). MPTP in urine will likely be ionized and not volatile, and be well absorbed by the animal bedding. Also, less than 0.01% of the total injected dose of MPTP is detected as volatile MPTP, which probably originates from the animals exhaling MPTP or from vapors from contaminated urine (Yang *et al.* 1988).

One day after an injection of radiolabeled MPTP to mice, most of the radioactivity is localized in the brain and the adrenal gland, while all other organs contain 50–75% lower amounts of radioactivity (Johannessen *et al.* 1986). Analysis of the radioactive species recovered from different organs and body fluids such as bile, urine, blood, and CSF demonstrates variable amounts of unmetabolized MPTP soon after injection, but by 12–24 h postinjection, essentially all of the radioactivity corresponds to MPP^+ (Markey *et al.* 1984; Johannessen *et al.* 1986).

From the above, it appears that the potential risks of exposure to MPTP are through direct contact with the animal, the animal cage inner surfaces, and its bedding material. There is minimal risk from exposure due to airborne or vapor-borne forms of MPTP. Although safety procedures, as outlined below, must always be followed, the period of maximal risk of MPTP contamination is from the moment of injection to the time that MPTP or its metabolites are no longer found in the excreta of treated animals; as a precautionary measure, we recommend extending the period of high-risk from 3 days to 5 days post-MPTP injection.

Personal protection

Prior to discussing MPTP preparation, injection and animal experimentation, it is necessary to discuss the issues of the recommended facility and personal protective equipment (PPE). As a rule, only investigators and/or staff members who are trained in handling hazardous agents and who are familiar with MPTP safety procedures and practices should prepare and administer MPTP, and monitor the animals during the high-risk period (i.e. 5 days post-MPTP injection). Of note, any staff member who undertakes these tasks should give informed consent and not be coerced into taking on MPTP-related duties. Moreover, it is strongly recommended that all aspects of the MPTP experiment, including storage and solution preparation, take place in a dedicated procedure room (for small animals) or area within the animal room (for large animals), and not in a regular laboratory. For personal safety, when using MPTP, researchers are required to wear the PPE described below, during the preparation of the MPTP solution, the injection period, and 5 days postinjection. Thereafter, regular laboratory attire as required to handle animals is sufficient.

It is important to emphasize that in laboratories committed to MPTP research, one cannot exclude that exposure to even trace amounts of MPTP over many years of the same investigator and/or staff member may have negative consequences. This is one more reason why a heightened standard of protection must be implemented for any individual involved in MPTP experiments.

Dedicated procedure room and area within animal room

All MPTP experiments including preparation of solutions must be performed in a procedure or animal room under negative-pressure because aerosols from MPTP and its metabolites can be generated from bedding, excreta and animal fur. All animals should be acclimated to the room for 4–7 days prior to any MPTP experiment to allow for monoamine stabilization before MPTP injection since monoamine level alterations may affect intragroup lesion reproducibility. The procedure or animal room should have a 12-h light-dark cycle, a bench with a working area, a sink, and be temperature-controlled. For small animals like mice, it should also be equipped with an animal rack to hold all of the cages and a fume hood. All furniture should be of stainless steel or of any material, except wood, that is acid-resistant and washable. All working surfaces including the fume hood and animal racks should be covered with materials that are absorbent on the face-up side and non-absorbent on the face-down side. The entire floor of the procedure room or working area in the animal room for large animals should be covered with plastic-backed absorbent sheets. A warning sign clearly stating 'Danger! MPTP Neurotoxin Use Area – Entry Restricted' must be posted on the outside of the procedure or animal room door. The room must be locked at all times and the animal care staff informed of the ongoing use of MPTP and its dangers. They must also be informed that this room is off limits unless allowed to enter by the responsible investigator.

This procedure room or designated procedure area should be completely equipped with all of the necessary supplies for the MPTP experiments. It should also contain a sharps disposal container clearly labeled as hazardous waste, a container lined with a hazardous waste disposal bag for solid waste (diapers, gloves, animal shavings, etc.), gloves, absorbent pads, paper towels, markers, weighing scales for animals and MPTP, sterile saline, syringes with needles, 1% bleach (sodium hypochlorite) solution in water, a strong biodegradable detergent, personal protective equipment (see below), and deprenyl (selegiline), an MAO-B inhibitor, for accidental exposure to MPTP. It is imperative that the material safety data sheet (MSDS) for MPTP, which is supplied by the manufacturer, be kept in the room. Thus, once in the room or area, there should be no need to exit during the injection period.

Personal protection equipment

PPE must be worn during all procedures involving MPTP, including during the 5 days post-MPTP injection. The PPE is far more important when injecting mice than monkeys as mice require significantly higher doses of MPTP. The PPE consists of a one-piece garment with an

attached hood, elasticized wrists and attached boots made of a lightweight, chemically and biologically inert, non-absorbent material that is tear-resistant and provides protection from airborne particles. This garment should be easy enough to get into and economical enough to throw away after one wearing. For example, coveralls made of Tyvek fabric with elasticized wrists and boots and an attached hood (Kapplar, Guntersville, AL, USA) can be used. A full-face respirator with removable HEPA filter cartridges that is fit-tested to the individual is preferred for facial and respiratory protection. Alternatively, a half-face air-purifying respirator with removable HEPA cartridges that is approved by the National Institute of Occupational Safety and Health (NIOSH)/Mine Safety Health Administration (MSHA) for respiratory protection against dusts that is fit-tested to the individual using the respirator can also be used. The respirator is re-usable and should be thoroughly wiped with 1% bleach solution then washed with detergent after each use; wipes must be disposed of in the hazardous waste container. Splash-proof goggles and double-layered nitrile under latex gloves complete the PPE attire. All items comprising the PPE attire can be obtained from a large general laboratory supply company. The office of environmental health and safety in any Institution where MPTP is to be used must be consulted for guidance in obtaining PPE attire for use with MPTP.

Housing

For small animals such as mice, disposable cages and accessories are strongly recommended as they permit incineration of waste without bedding changes. Covering cages with filter bonnets is recommended to significantly reduce both room contamination and cross contamination of other animals. Small animal cages should be placed on the animal rack in the procedure room prior to and during the five-day period post-MPTP injection. All injections must be performed in the fume hood in the procedure room.

For large animals such as monkeys, enclosed cages should be used. The base of the cage and the drop pan must be lined with plastic-backed absorbent pads.

MPTP storage and handling

MPTP can be purchased from several commercial sources. Unless specifically required, do not use MPTP as the free base, but only as the hydrochloride or other non-volatile salt conjugate. MPTP storage and handling must be restricted to the procedure room or designated area within the animal room. Minimize the use of large volumes, concentrated solutions, and handling of MPTP powder and never transport MPTP solutions or opened vials of MPTP outside of the dedicated room. MPTP may be purchased in small quantities of 10 mg or 100 mg in glass septum bottles. Vials of MPTP must be kept closed until used and stored at room temperature in a container within a vacuum-sealed desiccated container. This second container should be kept in a locked cabinet with a permanently affixed 'MPTP – Neurotoxin' label. This cabinet must be secured to a non-removable surface in the procedure room or area.

Only investigators appropriately trained in the handling of MPTP should perform manipulations involving the powder. Use of glass containers will reduce handling problems that result from the electrostatic properties of plastic. It is strongly recommended that a balance dedicated to weigh MPTP powder be kept in the procedure room. Prior to weighing MPTP powder, cover the weighing area with pads dampened with 1% bleach solution to reduce the risk of airborne MPTP powder particles. To minimize the risk of MPTP powder spills, the weighing procedure described by Pitts *et al.* (1986) is a safe method: tare a small container (e.g. small scintillation glass vial with a screw cap); take the tared container and place an approximated amount of MPTP in it, close and wipe container with 1% bleach solution; weigh container; then add solvent to give desired concentration; again wipe container and all other items with 1% bleach solution; dispose of all wastes in a hazardous waste container. Alternatively, if a given experiment requires a total daily dose of less than 10 mg or 100 mg of MPTP, then it is safer not to open the vial and weigh the powder but to add the desired volume of solvent/vehicle directly to the sealed 10 mg or 100 mg vial. It must be understood that this MPTP solution has to be used in one day and the remainder discarded since MPTP in solution oxidizes at room temperature; prior to discarding the used MPTP sealed vial, inject a volume of 1% bleach solution equivalent to the volume of MPTP solution remaining into the vial, then discard the vial as biohazardous liquid waste. We previously found that storing MPTP solution at −80°C retards its oxidation as MPTP solution appears stable up to 2 months (personal observation). However, unless one has a dedicated −80°C freezer for MPTP storage, other issues such as laboratory safety will arise and that even without mentioning the negative impact of thawing and freezing of MPTP solution on its neurotoxic potency.

Animals should be injected only with sterile solutions of MPTP prepared by either filtration through a disposable 0.22 μm filter unit or by dissolving the compound in sterile saline or water. Do not autoclave MPTP solutions, as this will vaporize the compound and may lead to exposure from inhalation.

Injection of MPTP

As mentioned above, a number of different injection regimens have been used to produce the desired MPTP lesions. These are based on a number of factors, including experimental design, degree of desired lesion, and species

used. As indicated, mice, which typically require greater amounts of MPTP to produce lesions, can be injected either subcutaneously or intraperitoneally, single or multiple injection, and with a wide range of concentrations. Whatever the regimen used, it is recommended that all MPTP injections to mice be performed in a fume hood. Vials from which MPTP is drawn should have a septum or be covered with parfilm to eliminate potential aerosols and spills and to avoid drops on the needle end. Change gloves frequently during the course of and at the end of the injection schedule. This will prevent any contamination of the PPE and decrease the possibility of overt contamination of equipment.

On the day of or on the evening before the experiment, all animals are weighed and coded. About a half-hour before starting the injection schedule, sterile MPTP solution should be prepared to the desired working concentration. During animal injection, care must be taken to avoid self-inoculation; special attention to animal restraint will significantly reduce this risk. For injection, place the mouse cage in the fume hood and when injecting, hold the animal so that any urine spray will fall into the cage and not on the surrounding areas, since mice, when held, tend to expel urine which can contain significant amounts of MPTP (Yang *et al.* 1988). Make sure the mouse is not held so tightly as to cause backflow of the injected MPTP from the peritoneum. Larger animals such as squirrel monkeys must be placed in restrainers for injection. It is not practical to inject large animals in a fume hood. Inspect injection site for leakage or spilled solution and wipe with a small pad dampened with 1% bleach solution. When discarding syringes, do not clip, recap or remove needles from syringes; fill the syringe with 1% bleach solution and then place the syringe with attached needle in a sharps container to be disposed of as biohazardous waste. At the end of the injection schedule, the remaining MPTP solution must be destroyed with an equivalent volume of 1% bleach solution as described above.

Cage changing

The greatest potential for exposure to MPTP and its metabolites is from contaminated bedding and caging immediately following MPTP injection and during the period that MPTP or its metabolites are likely to be in the excreta of treated animals. Therefore, when handling cages and their contents, it is important that the PPE be worn.

Used disposable mouse cages containing contaminated bedding should be dampened with 1% bleach solution and then be carefully placed into a plastic biohazard bag, tied off, and sent for incineration. When using re-usable cages, bedding should also be dampened with 1% bleach solution, then carefully placed in the biohazard bag, packaged and disposed of as biohazardous waste. Immediately after emptying re-usable cages, soak cages and accessories with 1% bleach solution for 10 min, rinse, then wash with detergent and rinse thoroughly with water. Mouse cages may then be sent to central cage washing facilities. The absorbent material that covered the rack surfaces should be sprayed with 1% bleach solution, allow to soak for 10 min and then disposed of as hazardous waste. For large animal cages, spray plastic-backed absorbent pads that line the cage bottoms and drop pans with 1% bleach solution, allow to soak for 10 min, then remove pads and place them in the biohazardous waste container; replace used linings with fresh pads. This needs to be done on a daily basis. Wash cages and accessories thoroughly with 1% bleach solution, rinse, then wash with detergent and rinse thoroughly with water. The procedure described above assumes that MPTP-injected animals remain in the same cage for 5 days postinjection and change out should occur only after the 5 days postinjection period. In the case of prolonged MPTP exposure protocols (i.e. weeks to months), while the procedure room or area will remain off-limits throughout the treatment period (plus the five days postinjection period), for mice, change only cage bottoms once a week following the procedure described above and, for monkeys, it is advisable to move monkeys to clean cages every other week and to handle the dirty cages as described above.

Counter tops in the procedure room or area should be cleaned with 1% bleach solution. Floor coverings should be carefully removed and disposed of as hazardous waste. Routine animal care can be re-instituted five days post last MPTP injection and once the procedure room or area has been cleaned by the responsible investigator and/or staff member.

Animal tissues

Potential risk of exposure to MPTP or MPP^+ may occur when animals are killed for tissue collection up to 5 days following MPTP administration. During this period, mice should be killed in the fume hood and the appropriate PPE worn by the researcher during blood and tissue harvesting procedures. All working surfaces are lined with plastic-backed absorbent pads, which should be changed if stained with body fluids. Since decapitation is the primary method of killing for small animals in MPTP studies, care should be taken to prevent blood spatters, and urine and feces should be contained. Brain tissues are best dissected on an inverted glass Petri dish covered with water-dampened filter paper and placed on regular ice. All instruments, including the Petri dish used for dissection, should be soaked in 1% bleach solution for 10 min, rinsed, then washed with detergent and rinsed with water. Collected tissues should always be handled with double gloves, and brain remnants and the remaining carcass, which may contain MPTP and

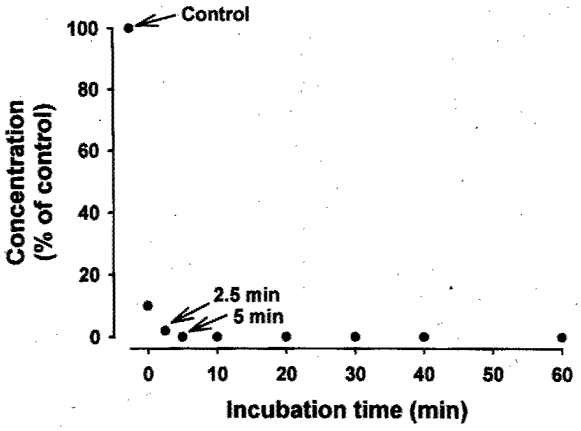

Fig. 1 Time-dependent effect of 1% bleach solution on MPTP. A 5-mg/mL of MPTP-HCl solution in saline was incubated at room temperature for different lengths of time with 1 volume (v/v) of 1% bleach (sodium hypochlorite) solution in water. After the indicated time of incubation, an aliquot of the mixture was injected into an HPLC-UV system and MPTP levels were quantified as described (Przedborski *et al.* 1996).

metabolites (Yang *et al.* 1988), must be discarded following biohazardous waste practices for animal waste.

For the perfusion of small animals, a grid overlaying a collection pan works best. Thus, blood and perfusion solution will be collected in the pan and can then be poured into a bottle or can be discarded as biohazardous waste. As per proper biohazardous waste disposal, the outside of the waste container must be wiped with 1% bleach solution.

For the perfusion of large animals, plastic tubing should be attached to the drain of the dissection table and a liquid biohazard waste container. This will catch any perfusion solution and prevent contamination of the water system. The collected perfusate will be discarded as biohazardous waste. After the perfusion procedure, the table must be washed with 1% bleach solution, rinsed, then washed with detergent and rinsed with water.

Decontamination, cleaning, and disposal

Often, one may see that 0.1 M HCl is used for cleaning up following MPTP experiments. However, we have HPLC evidence showing that HCl, up to 2 M and after incubation for more than 1 h at room temperature, does not destroy MPTP at all. Conversely, a 5% potassium permanganate solution in water completely destroys MPTP almost immediately. However, since potassium permanganate is such a powerful oxidant, it can produce hazardous exothermic reactions with several compounds like detergents and must be neutralized with ascorbic acid prior to being discarded as non-toxic waste. We have also found that bleach is as efficient as potassium permanganate in destroying MPTP, yet more friendly to use as it does not cause dangerous reactions with detergents and does not require specific treatment prior to discarding. Bleach is commercially available as a 5–10% stock solution. It can be readily diluted to the desired concentration with water and kept at room temperature indefinitely. Using a 1% bleach solution in water, which corresponds to twice the Environmental Protection Agency (EPA) recommended concentration for disinfection, we found that the action of bleach on 5 mg/mL of MPTP–HCl in saline is rapid in that after 5 min, at room temperature, there is no longer any detectable MPTP (Fig. 1). The 'almost' instantaneous destruction of MPTP by the bleach solution, as illustrated in Fig. 1, is not a surprising finding since the bleach-mediated reaction corresponds not to an enzymatic reaction but to a straight biochemical oxidation. In addition, we found that 10 min incubation of 5 mg/mL MPTP–HCl with different concentration of bleach solutions, ranging from 0.5 to 2.5%, had similar effects on MPTP. Therefore, our recommendation for MPTP decontamination is 10 min of soaking in 1% bleach solution. In contrast to their effects on MPTP, neither 2.5% bleach solution nor 5% potassium-permanganate destroyed MPP^+, even after an overnight incubation. This is not surprising, as MPP^+ is notoriously stable and resists destruction even after exposure to extremely harsh chemical and physical treatments. High doses of MPP^+ administered systemically (i.e. 25 mg/kg intraperitoneal) to mice produce oxidative damage to the lung, but fail to affect the nervous system (Johannessen *et al.* 1985). This is consistent with our observation that the intraperitoneal or subcutaneous injection of different doses of radiolabeled and non-radiolabeled MPP^+ to mice failed to show any accumulation of radioactivity in the striatum or to produce any damage to the dopaminergic systems of the brain (unpublished observation). Nevertheless, the direct injection of MPP^+ into the striatum does produce dopaminergic neurotoxicity (Giovanni *et al.* 1994b). These data indicate that the work-related hazards of MPP^+ involve peripheral organs such as the lungs and then only if high amounts reach the blood stream or the respiratory tract. Therefore, MPP^+ is far less hazardous than its parent compound and thus the real safety goal is the destruction of MPTP.

Only investigators appropriately trained in the handling of MPTP should clean up spills. Prior to any decontamination procedure, determine the maximum quantity of MPTP involved in the spill and the location of the spill.

If the room is properly maintained as stated above, linings and underpads will catch any spills. In case a liquid spill does occur, wearing the PPE, the researcher should immediately spray the linings and underpads with 1% bleach solution, allow to soak for 10 min, then remove, and place these in hazardous waste disposal bags. In the event that pads and linings have not caught all of the spill, absorb MPTP spill with absorbent plastic-backed pads to prevent

MPTP solution from contaminating gloves and discard as hazardous waste. The dry area is then soaked with 1% bleach solution, rinsed with water, then washed several times with detergent, rinsed with water, and dried with pads. Discard these materials in hazardous waste bags as well. Recover work area and inform the environmental health and safety office that an MPTP spill has occured and what measures were used to remove that spill.

To clean up MPTP powder spills, cover with a disposable towel dampened with 1% bleach solution, then pick up all materials and put into a hazardous waste container. Then, soak the area with 1% bleach solution, rinse with water, then wash several times with detergent, rinse with water, and dry with pads. Discard these materials in hazardous waste bags. Recover area, then inform the environmental health and safety office that a MPTP powder spill has occured and what measures were taken to contain and clean up the powder spill.

If clothes become contaminated with MPTP, immediately remove clothing and shower. After obtaining fresh clothing, report directly to a medical service. A very careful evaluation of any potential MPTP exposure is critical (see medical emergency and surveillance). Persons assisting exposed individuals should wear the PPE attire.

Plan experiments to avoid generating large quantities of contaminated glass or metal; these materials are difficult to incinerate, and large quantities can create waste disposal problems. Contaminated glass and metal can be decontaminated using 1% bleach solution followed by detergent washes and rinses. Decontaminate all equipment with wipes dampened with 1% bleach solution before repair work is performed, before transferring equipment to other operations, and before discarding. Pay special attention to internal parts of equipment that may have become contaminated.

Prevention, medical emergency and surveillance

To date, there has been no report in the literature of the inadvertent exposure of a researcher to MPTP while conducting MPTP experiments. A single report of a research chemist who suffered a fatal exposure to large amounts of MPTP during its synthesis has been documented and represents the only inadvertent human exposure to MPTP (Langston and Ballard 1983). However, despite the safe track record of MPTP use, precautionary emergency procedures must be employed to avoid potential injury from acute exposure to the toxin (such as a needle prick).

As indicated above, MAO-B inhibitors prevent the conversion of MPTP to its toxic metabolite, MPP^+ thereby preventing neurotoxicity. For example, pretreatment of animals with deprenyl, a potent irreversible MAO-B inhibitor prevents MPTP-induced neurotoxicity (Cohen *et al.* 1984; Mytilineou and Cohen 1985; Fuller *et al.* 1988). On the other hand, except for a single report (Tatton 1993), there is no evidence that MAO-B inhibition by deprenyl or by other compounds, following exposure to MPTP provides any neuroprotection. However, in case of accidental exposure to MPTP, in an attempt to block the conversion of any remaining MPTP to MPP^+ it is recommended that deprenyl be administered immediately. As far as we know, there is no established deprenyl regimen for accidental exposure to MPTP. Since the goal here is to prevent the conversion of MPTP by inhibiting MAO-B, as rapidly and profoundly as possible, we suggest an initial large dose of deprenyl (e.g. four 5 mg tablets) be taken orally at once. Although it may be prudent to continue deprenyl medication (e.g. 5 mg twice a day) for some time, it is unknown whether this is justified. Short-term surveillance is necessary for the appearance of hypotension from the deprenyl or the development of acute parkinsonian symptoms from the MPTP exposure. In addition, following the administration of a large dose of deprenyl, individuals must be cautious in consuming tyramine-containing foods (i.e. cheese) and in taking medications containing pharmacologically active amines. Prior to beginning any MPTP investigation, deprenyl must be available for emergency use and must be kept in a closed container at all times in the procedure room or area for immediate use, if necessary. Furthermore, it is advisable that individuals who are planning to embark upon a series of MPTP experiments consider a treatment of 5 mg twice a day of deprenyl prior to (e.g. 3–5 days before) and during the experiments. This may be especially indicated for a person first learning the protocol or if there is an increased risk of contact with MPTP. This should be done only after consulting one's personal physician.

Conclusion

To date, MPTP remains the best experimental model of PD. To this end, it is extensively used in various animal species and especially in mice. However, even as a research tool, MPTP is an extremely hazardous compound, which can be injected, ingested, inhaled, and/or absorbed. Because of its demonstrated toxicity to humans, the use of MPTP among researchers is a serious concern. Over the years, a better understanding of the physicochemical properties of this toxin, its metabolism, and its body distribution has enabled investigators to develop practices and procedures for the safe use of this compound. These include improved procedures for preparing MPTP solutions and for its injection into animals, proper protective equipment, reducing potential exposure from animal excreta, proper decontamination and disposal procedures, and medical treatment and surveillance in case of accidental exposure. Despite the fact that we have tried to cover the most common situations and topics related to MPTP use, this review cannot cover all possible aspects of the safe use of

this hazardous compound. Accordingly, there can be no substitute for common sense and proper laboratory practices in the use of dangerous compounds such as MPTP. It is hoped, however, that this review has built upon the guidelines presented by others in the past and, in conjunction with our recent knowledge of MPTP, will lead to the effective and safe use of the MPTP animal model of PD.

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A Supplement to *Trends in Neurosciences* Vol.24 No.11

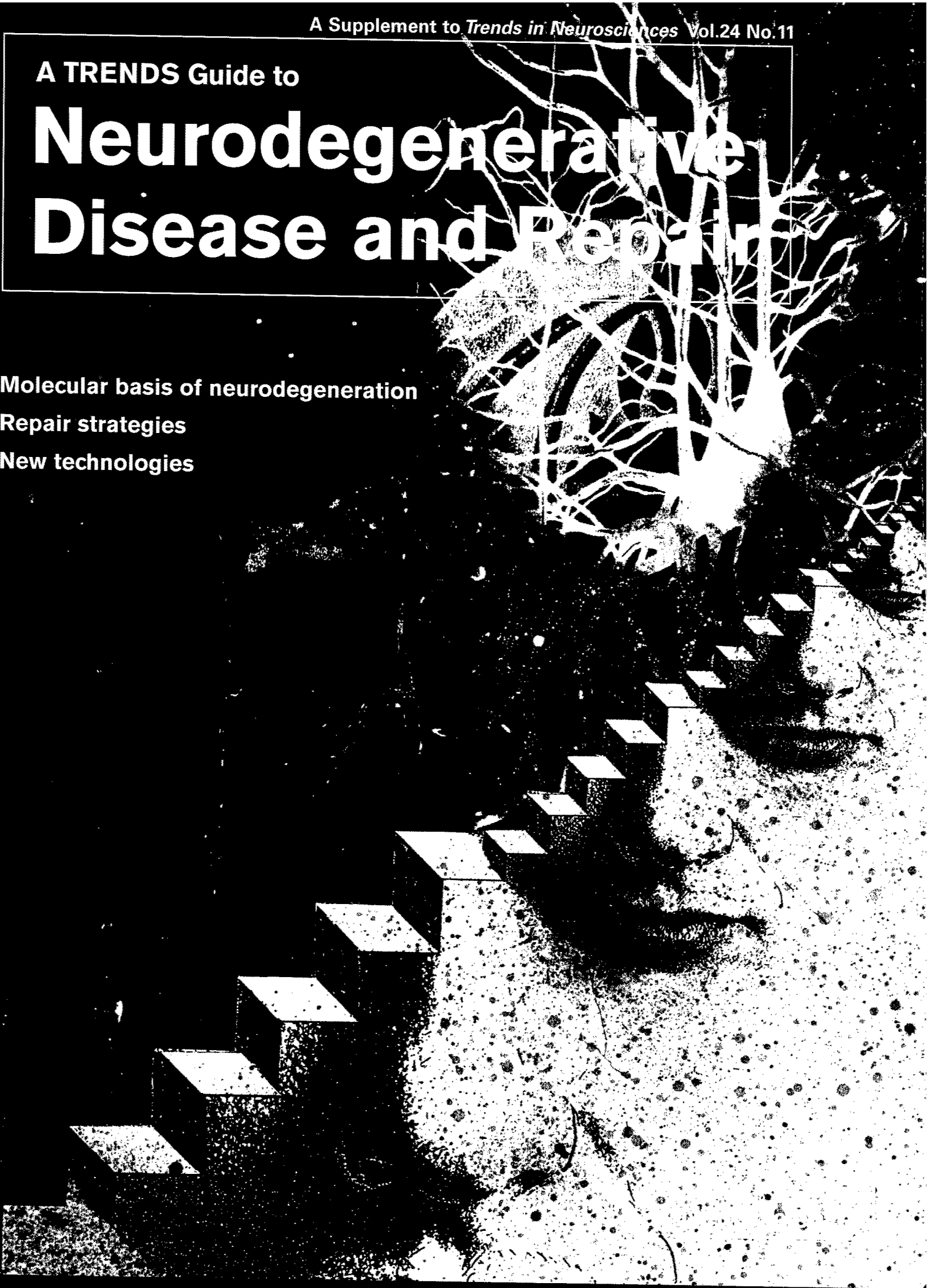
A TRENDS Guide to

Neurodegenerative Disease and Repair

Molecular basis of neurodegeneration

Repair strategies

New technologies



Cover image

Neurodegenerative disease is characterized by a progressive loss of function in either motor or cognitive ability; this decline is represented artistically on the cover by the reducing size of the face as the staircase gets higher. Research into neurodegeneration can involve attempting to understand molecular mechanisms underlying the diseases or developing novel therapies. However, whether at the molecular level or macroscopic level, all our research efforts have as their ultimate aim the improvement of human quality of life.

A TRENDS Guide to Neurodegenerative Disease and Repair

Contents

The disciplines involved in research into neurodegeneration are diverse, ranging from cell biology and biochemistry to behavioural neuroscience. The tremendous advances in our understanding that have been gained in recent years through these various disciplines have inevitably led to opportunities for therapy and repair. Some of the most recent and important developments are collected together in this special supplement to *Trends in Neurosciences*. We hope that you enjoy this supplement, and welcome any feedback that you have.

Siân Lewis
Editor, *Trends in Neurosciences*

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Editorial

A recent estimate suggests that the number of people over 65 years-of-age in the USA will double between now and the year 2050. Clearly, our population is aging, and aging brings with it the increased likelihood of neurodegenerative disease (ND). As a consequence, one of the great challenges of biomedical research will be to develop successful therapeutic strategies to enable us to enjoy our old age – not just to live longer, but to live longer *in health*.

The field of NDs is too vast to be able to cover each and every topic in this special issue, but our aim was to discuss important recent advances and highlight some of the more encouraging therapeutic approaches.

Protein accumulation and potential therapeutic targets

The most common forms of age-related NDs are Alzheimer's disease (AD) and Parkinson's disease (PD), and both are characterized by abnormal protein aggregation. AD is characterized by the formation of Aβ-containing plaques, which involves γ-secretase cleavage of a precursor protein. The identity of this enzyme is currently uncertain, but presenilins are popular candidates. On pages S2–S6, Sangram Sisodia discusses the evidence for and against presenillins, and describes how their role in the CNS is more complex than we originally thought.

An important question is why these toxic proteins accumulate – why are they not eliminated? It is emerging that an overwhelmed ubiquitin-dependent proteolysis system might well be responsible, as described by Ted Dawson (pages S7–S14).

Insights from molecular analysis

Clearly, many NDs involve both genetic and environmental factors, and transgenic animals are one of the most widely used methods for studying genetic mutations and their pathological effects. Serge Przedborski outlines the major areas of progress resulting from the numerous animal models of Parkinson's disease, in which some of the more detailed aspects of the disease are revealed (pages S49–S55).

Two other NDs for which the molecular basis is beginning to emerge are Huntington's disease (HD) and amytrophic lateral sclerosis (ALS), a progressive ND of motor neurons. HD is characterized by a CAG expansion that encodes a polyglutamine tract, which results in progressive protein accumulation. Cynthia McMurray's pioneering work in discovering the mechanism by which the CAG expansion occurs (see pages S32–S38) offers a new potential therapeutic target with implications beyond HD itself. And on pages S15–S20, Joe Beckman explains how zinc deficiency in the superoxide dismutase enzyme might underlie most, if not all, cases of ALS.

Repair strategies

What is the best way to target the genetic abnormalities when we become aware of them? Antisense technology might be one solution, and despite shaky beginnings the continued commitment to research into this approach is beginning to yield results. The efforts to reduce toxicity and improve specificity and efficacy have resulted in a gene therapy approach with real promise. The potential of this approach, applied specifically to neurons, is discussed by Peter Estebeiro on pages S56–S62.

It is hoped that a greater understanding of ND will lead to new therapies; however, one of the most exciting new approaches to AD treatment has resulted from the serendipitous observation that patients treated with cholesterol-biosynthesis inhibitors had a lower incidence of AD. Tobias Hartmann describes the subsequent clinical trials of these drugs as a primary therapy for AD and the highly encouraging results that have been obtained (see pages S45–S48).

Although halting the progress of an ND is good, *reversing* the pathology of the disease is better, and after decades of research, some genuinely exciting approaches are beginning to emerge. Multiple sclerosis, a demyelinating disease that affects the peripheral nervous system (as well as the CNS) is a complex disease involving abnormal interplay between the immune and nervous systems. On pages S39–S44, Moses Rodriguez describes how it might be possible to activate selectively the endogenous repair mechanisms that stimulate remyelination.

Normal and abnormal aging – is there anything we can do?

There is certainly no panacea to the manifest problems of neurodegeneration, but in their fascinating article, Tom Prolla and Mark Mattson discuss the surprisingly dramatic effects observed in rats fed on a diet where the energy intake is 70% of normal (see pages S21–S31). These animals have vastly improved lifespans, retain the physiology of young animals for longer and might have increased resistance to ND; an interesting irony given the rising levels of obesity in modern society.

And so I hope you will enjoy the articles in this special issue, and please remember to visit *Trends in Neurosciences* regularly for its continued coverage of this field and fresh insights into research advances as they occur.

Siân Lewis
Editor,
Trends in Neurosciences

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γ-Secretase: never more enigmatic

Sangram S. Sisodia, Wim Annaert, Seong-Hun Kim and Bart De Strooper

It is widely believed that the pathogenesis of Alzheimer's disease (AD) is intimately, if not causatively, associated with the deposition of ~4 kDa β-amyloid (Aβ) peptides in the cerebral cortex and hippocampus of affected individuals. Aβ peptides are liberated from transmembrane proteins, termed β-amyloid precursor proteins (APP), by the concerted action of β- and γ-secretase(s). Whereas the identity of β-secretase is no longer in question, the identity of γ-secretase, which is responsible for the intramembranous processing of APP, has never been more enigmatic. Considerable evidence has accrued to impugn the presenilins (PS) as the executioners of intramembranous processing of APP. Here, we summarize these observations and review recent evidence that argues against the prevailing hypothesis that PS function as γ-secretases.

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Alzheimer's disease (AD), the most common cause of dementia in the elderly, is a progressive neurodegenerative disease associated with impairments in cognition and memory. AD is associated with several risk factors, including age and inheritance. The majority of early-onset cases of AD are familial (FAD) and are inherited as autosomal dominant disorders; mutations in genes encoding homologous, polytopic membranes, termed presenilin 1 (PS1) and presenilin 2 (PS2), and the type I membrane protein, amyloid precursor protein (APP), cosegregate with affected members of FAD pedigrees[1]. In all cases, expression of mutant PS1, PS2 or APP leads to the enhanced production of highly fibrillogenic $A\beta_{42}$ peptides, which are deposited selectively in senile plaques in the amygdala, hippocampus and neocortex. Aβ peptides are liberated from APP by the concerted action of proteolytic enzymes termed β-and γ-secretases. A membrane-tethered, aspartyl protease called BACE1 is certainly responsible for generating the amino-terminus of Aβ peptides (reviewed in Ref. 2). However, the identity of γ-secretase, which catalyzes the hydrolysis of APP within its transmembrane segment, has been an enigma. This review highlights the salient observations that have lent support for the notion that PS are γ-secretase, and offers alternative models for PS function that are inconsistent with a direct mode of action of these polypeptides as the catalytic entities involved in intramembranous proteolysis of APP and Notch1.

PS: a primer

PS and their *Caenorhabditis elegans* homologs, SEL-12 and HOP-1 (Refs 3,4), are polytopic membrane proteins that accumulate ~27–30-kDa N-terminal fragments (PS-NTFs) and ~16–20-kDa C-terminal fragments (PS-CTFs) derivatives (see Ref. 5 for review). The accumulation of PS-NTFs and PS-CTFs is highly regulated and saturable (that is, the levels cannot exceed endogenous levels)[5], but the components of the apparatus responsible for establishing PS levels have not yet been determined. Nevertheless, the PS-NTF and PS-CTF remain associated in high molecular weight complexes that range from ~100–150 kDa (Refs 6,7) to $>2 \times 10^6$ Da (Ref. 8). These polypeptides are found predominantly in the endoplasmic reticulum (ER) and in ER–Golgi transitional compartments (ERGIC)[9–11].

PS as γ-secretase: setting the scene

The first act: APP and pathogenic Aβ peptides

The demonstration that FAD-linked PS1 or PS2 alter APP processing in a manner that leads to elevated production

of highly fibrillogenic $A\beta_{42}$ peptides (reviewed in Ref. 1) provided the first hint of a role for PS in γ-secretase processing of APP. However, the requirement of PS in intramembranous γ-secretase processing of APP was not fully appreciated until the discovery that cells with a genetic ablation of PS1 (Refs 12,13) or cells with compound ablation of both PS1 and PS2 (Refs 14,15) exhibit marked reduction, or complete loss, of Aβ peptide production, respectively. In parallel, intracellular accumulation of α- and β-secretase-generated APP carboxyl-terminal fragments (APP-CTFs) (Refs 12–15) in these settings led to the inescapable conclusion that the membrane-tethered APP-CTFs are the penultimate precursors of Aβ peptides and that PS are essential mediator(s) of the γ-secretase processing event.

The second act: Notch1 and NICD

In what must be viewed as a pure stroke of scientific serendipity, genetic epistasis studies of the *C. elegans* and *Drosophila melanogaster* PS homologs, and phenotypic analysis of PS-deficient mice (see Ref. 16 for review), strongly suggest that PS plays a crucial role in facilitating developmental signaling of the lin12/glp1/Notch1 family of receptors. Notch1 is subject to proteolytic processing reactions not dissimilar to APP: plasma-membrane-associated Notch1 species are first cleaved within the ectodomain by a disintegrin and metalloprotease domain (ADAM)-like protease, and subsequently processed by a γ-secretase-like activity within the transmembrane segment. The latter reaction liberates a cytoplasmic Notch1 derivative called Notch intracellualar domain (NICD), which is translocated to the nucleus and functions as a transcriptional coactivator[16,17]. NICD production is largely, albeit incompletely, abrogated in PS1-deficient cells[18], but entirely eliminated in cells with compound deletions of both PS1 and PS2[14,15]. Collectively, the genetic and biochemical evidence reinforces the notion that PS are necessary for γ-secretase processing of APP and Notch1.

The third (and final?) act

The proposal that PS1 are γ-secretases gained considerable momentum when Wolfe and colleagues reported that expression of PS1 harboring alanine substitutions of two conserved aspartate residues at positions 257 or 385 compromised Aβ secretion and induced accumulation of CTFs; biochemical phenotypes that largely mimic the PS1-deficient state[19]. These observations were particularly intriguing because both aspartate residues are thought to reside within transmembrane domains six and seven, supporting the model that PS1 are unusual diaspartyl proteases that catalyze intramembranous proteolysis of APP (Ref. 19).

Although the reported functional role of PS aspartate residues in modulating γ-secretase remains somewhat puzzling, the biochemical evidence supporting the notion that presenilins are γ-secretases is quite compelling. Li and colleagues[8] demonstrated that PS1 is intimately associated with protein complexes of ~2 × 10^6 Da, where the entirety of γ-secretase activity resides. Moreover, the γ-secretase activity from these complexes was inhibited by L-685,458, a potent aspartyl protease 'transition-state' analog. Remarkably, a photoactivatable analog of L-685,458 specifically labeled the PS1 or PS2 derivatives[20], the findings of which were confirmed by Esler and colleagues, who employed a different class of 'transition-state' analog inhibitors of γ-secretase[21]. In this regard, Steiner *et al.*[22] have noted sequence similarities between a PS sequence that includes the crucial aspartate 385 residue and a domain of the polytopic membrane proteases called bacterial type 4 prepilin peptidases (TFPP)[23].

γ-Secretase and PS: the caveats

In view of the strengths of the collective genetic and biochemical evidence, one might be inclined to conclude that PS are γ-secretases[24]. However, as we review next, a wealth of data have recently emerged to cast a veil of doubt on these conclusions.

Is PS1 the executioner? We begin by commenting on the substrate selectivity of 'γ-secretase' and the presumed role of PS in proteolysis (Fig. 1). Whereas in the case of Notch1 a valine residue four amino acids before the end of the Notch1 transmembrane segment occupies the P1′ position[25], the principal Aβ peptide, $A\beta_{40}$, is generated following cleavage between the twelfth and thirteenth amino acids of the APP transmembrane domain. In contrast to the highly selective sequence requirement and precise site of Notch1 processing for the generation of NICD (Ref. 25), the production of $A\beta_{40}$ or related peptides is largely insensitive to the sequence at, or flanking, the scissile bonds[26]. Moreover, a family of Aβ-related peptides with termini at 34, 38, 39, 42 and 43 are detected in conditioned medium and brains of transgenic mice[27,28] (Fig. 1). Despite these differences in the precise sites of proteolysis within the APP and Notch1 transmembrane domains, the γ-secretase inhibitor, L-685,458 inhibits production of the NICD and Aβ derivatives equally[29]. These data suggest that, if γ-secretase is a single entity, it must be highly unusual. In this regard, whereas the vast majority of the reported FAD-linked PS missense mutations (detailed at http://molgen-www.uia.ac.be/ADmutations/) occur within transmembrane helices, many do not. It is possible that the mutations in transmembrane domains are distributed on a single face of their respective helices[30]. Might the transmembrane

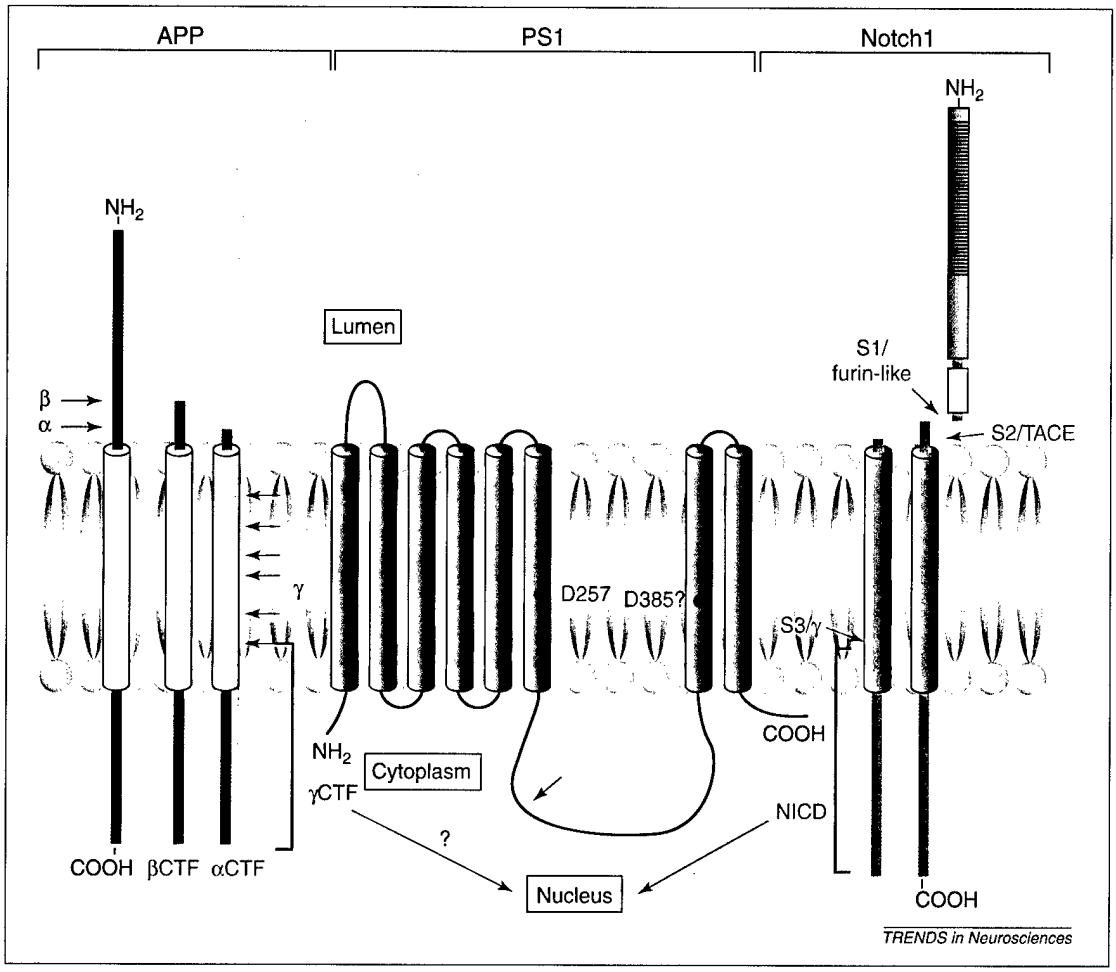

Figure 1. Schematic representation of proteolytic cleavages of β-amyloid precursor proteins (APP) and Notch1

The figure depicts the eight transmembrane domain topology of presenilin 1 (PS1; blue) as well as the type 1 membrane topology of APP (yellow) and Notch1 (red). The endoproteolytic cleavage site of PS1 is indicated by the red arrow, and the proposed crucial aspartates (D257 and D385) of PS1 are indicated as a red dot. The intramembranous cleavage sites in APP and Notch1 are indicated by blue arrows, and regions corresponding to γ-carboxyl-terminus fragments (CTF) and the cytoplasmic Notch1 derivative Notch intracellular domain (NICD) are also indicated (green arrows). Note that APP is cleaved at multiple sites by γ-secretase, whereas cleavage of Notch1 occurs at a single site. For details, see the text and the references therein. The pink arrow indicates the furin-like proprotein convertase site. The light blue arrow indicates the TACE site and the black arrows indicate the α and β secretase sites in the APP ectodomain. Abbreviation: TACE, tumor necrosis factor α converting enzyme.

domains of PS surround a central canal wherein APP-CTFs are subject to proteolysis? Although attractive, this suggestion does not explain why, independent of the site of mutation along the polypeptide backbone, every FAD-linked PS variant enhances processing at only a single site ($A\beta_{42}$) in the APP transmembrane domain. Equally confusing is that whereas expression of the FAD-linked L166P PS1 variant or experimental L286E or L286R PS1 mutants[31] leads to overproduction of $A\beta_{42}$ peptides, these PS1 variants are incapable of supporting the production of NICD.

In toto, these experiments unequivocally prove that γ-secretase processing of APP and Notch1 can be dissociated, leading us to conclude that the catalytic activities responsible for processing these substrates are not one and the same.

The notion that PS are diaspartyl proteases emerged from studies showing that mutagenesis of two aspartyl residues (aspartate 257 and aspartate 385) within proposed transmembrane domains six and seven (Fig. 1) leads to inhibition of Aβ production[19]. However, there is neither complete agreement that the domain that includes aspartate 385 of PS1 exists as a membrane-spanning helix nor that aspartate 257 plays a role in Aβ production[32]. Furthermore, whereas PS1 harboring a D385A substitution abrogates Aβ production, PS1 with alanine substitutions at both aspartate 257 and aspartate 385 does not (Seong-Hun Kim and Sangram S. Sisodia, unpublished). Remarkably, the D257A, D385A and compound D257A–D385A PS1 variants completely eliminate NICD production. The most parsimonious explanation for these

results is that the γ-secretase activities responsible for processing APP and Notch1 are distinct and differentially sensitive to the expression of the PS1 aspartate variants. Indeed, it now seems clear that whereas PS polypeptides exist in larger, heteromeric complexes, expression of aspartate PS1 mutants reduces the size of the PS complex[33]. These findings suggest that the aspartate residues are crucial for assembly of the functional γ-secretase complex, rather than for promoting catalysis *per se*. Indeed, recent studies have demonstrated that expression of PS1 with substitutions of a proline residue in the PS1-CTF or deletion of transmembrane domains 1 and 2 (regions that are remote from the two crucial aspartate residues), also effect APP metabolism in a manner similar to the PS aspartate variants[34,35]. Hence, the conclusion that the aspartate residues play a central role in intramembranous proteolysis of APP might need to be revised.

Resolution of the paradox: PS as molecular chaperones

The most unsatisfying feature of the proposal that PS are γ-secretase is that the subcellular distributions of PS tend not to overlap with the cellular sites of Aβ and NICD production. As noted earlier, PS are predominantly resident in early compartments of the secretory apparatus, but NICD is generated at, or near, the plasma membrane[36]. Moreover, α- and β-secretase generation of APP-CTFs are obligatory for γ-secretase processing, and these reactions occur in subcellular compartments distal to the ER and include the Golgi, endosomes and plasma membrane[37,38]. Attempts to resolve this 'spatial paradox'[11,38] might be futile: it has been argued that limiting (and, by definition, undetectable) levels of PS are recruited to late secretory compartments where they act as γ-secretase. By contrast, and in view of the preponderant localization of PS in the ER and/or ERGIC, we offer the alternative hypothesis that PS is essential for the assembly and trafficking of APP, the APP-CTFs and/or the γ-secretases. Supporting this view, the trafficking of APP, the tyrosine kinase receptor TrkB, and a PS1-interacting protein called ICAM-5 (telencephalin) to the cell surface, is altered in primary neurons from PS1-deficient mice (Ref. 13 and Wim Annaert and Bart De Strooper, unpublished observations). Our proposal that PS plays a broader role in facilitating the trafficking of γ-secretase or membrane-tethered substrate of γ-secretase to appropriate cleavage and/or degradation compartments is not inconsistent with the model posited earlier for the delivery of the membrane-anchored sterol-regulatory-element-binding protein (SREBP) protein to the Golgi by the ER-resident polytopic membrane protein SREBP cleavage-activating protein (SCAP)[39].

Concluding remarks

Despite overwhelming experimental support for a role of PS1 and PS2 in γ-secretase processing of APP and Notch1, a portrayal of PS as γ-secretase now appears far too oversimplified. It is our view that these polypeptides are unlikely to be the sole effectors of intramembranous proteolysis of APP and Notch1. The demonstration that nicastrin, a PS1-associated molecule, appears to modulate Aβ production supports this view[40]. Obviously, functional reconstitution of γ-secretase activity using purified components is the gold standard – a condition not yet achieved. This might be a rather challenging proposition, given the large, multimeric nature of the PS complex. Nevertheless, we remain optimistic that the entire repertoire of polypeptides in the presenilin complexes will soon be identified and that a reconstitution assay for γ-secretase activity will be developed. The results of these efforts will provide the foundation for a comprehensive assessment of the role of presenilins in the modulation of intramembranous, γ-secretase processing of APP and Notch1.

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The role of the ubiquitin-proteasomal pathway in Parkinson's disease and other neurodegenerative disorders

Kenny K.K. Chung, Valina L. Dawson and Ted M. Dawson

A unifying feature of neurodegenerative diseases is the abnormal accumulation and processing of mutant or damaged intra- and extracellular proteins; this leads to selective neuronal vulnerability and dysfunction. The ubiquitin-proteasomal pathway (UPP) is poised to play a central role in the processing of damaged and toxic proteins by ubiquitin-dependent proteolysis. The UPP can be overwhelmed in several neurodegenerative diseases. This results in the accumulation of toxic proteins and the formation of inclusions, and ultimately to neuronal dysfunction and cell death. Further analysis of the cellular and molecular mechanisms by which the UPP influences the detoxification of damaged and toxic proteins in neurodegenerative diseases could provide novel concepts and targets for the treatment and understanding of the pathogenesis of these devastating disorders.

Most, if not all, neurodegenerative diseases are marked by the presence of protein aggregates or inclusion bodies[1]. These include the prion protein (PrP) plaques in Prion disease, amyloid plaques and neurofibrillary tangles in Alzheimer's disease (AD), Lewy bodies in Parkinson's disease (PD) and dementia with Lewy bodies (DLB), nuclear inclusions in the poly-glutamine repeat diseases such as Huntington's disease (HD), spinocerebellar ataxias (SCA), dentatorubral and pallidoluysian atrophy (DRPLA), as well as other neurodegenerative diseases (Table 1). The linkage of two genes within the ubiquitin-proteasomal pathway (UPP) in hereditary PD (Refs 2,3), and recent advances in other neurological disorders, clearly indicate that the UPP plays a crucial role in the pathogenesis of neurodegenerative diseases, and has elevated the importance of the UPP in these disorders.

The close relationship between neurodegeneration and the ubiquitin system has long been implicated through the consistent findings of ubiquitin-positive protein aggregates in various neuropathological studies. In fact, the observation of ubiquitinated-protein inclusion bodies is one of the hallmarks of neurodegeneration. One general idea is that, under certain adverse conditions [including oxidative stress, protein misfolding during endoplasmic reticulum (ER) stress and aging], damaged proteins can accumulate in the cell. In addition, abnormal accumulation of proteins could occur owing to altered post-translational modification of newly synthesized proteins, abnormal proteolytic cleavage, diminished clearance of degraded protein and/or improper expression or altered gene splicing. The UPP might play a prominent role in the detoxification and targeting of damaged proteins for degradation. Under some conditions, the protein damage could be so severe that the clearance of damaged protein by the UPP and other degradative pathways might not be able to cope with the demand, resulting in the accumulation of damaged ubiquitin-tagged proteins and ultimately neuronal dysfunction and/or death.

Neurodegeneration and the UPP

There is increasing interest in the UPP in relation to the control of various important cellular processes. The system was first studied in reticulocyte lysates, which later resulted in the discovery of a pathway that provides

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Table 1. Representative neurodegenerative diseases that have ubiquitin-positive protein aggregates or inclusion bodies[a,b]

Disease	Gene	Mutations	Pathology	Ubiquitin-positive inclusion?
AD	*APP* *PS1* *PS2*	Missense	Amyloid plaques, neurofibrillary tangles	Yes
FTDP	*Tau*	Missense	Tau inclusions	Yes
Pick's	*Tau*		Pick bodies	Yes
ALS	*SOD*	Missense	Lewy-body-like inclusions	Yes
PD	*α-Synuclein* *UchL1* *Parkin*	Missense	Lewy bodies	Yes
DLB	*α-Synuclein*		Lewy bodies	Yes
MSA	*α-Synuclein*		Glial cytoplasmic inclusions (GCIs)	Yes
Prion	*Prion*	Missense	Prion protein (PrP) plaques	Yes[58]
DRPLA	*Atrophin 1*	Polyglutamine	Nuclear inclusions	Yes
HD	*Huntingtin*	Polyglutamine	Nuclear inclusions	Yes
SCA1	*Ataxin1*	Polyglutamine	Nuclear inclusions	Yes
SCA3/MJD	*Ataxin3*	Polyglutamine	Nuclear inclusions	Yes
SCA7	*Ataxin7*	Polyglutamine	Nuclear inclusions	Yes

[a]Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; APP, amyloid precursor protein; DLB, dementia with Lewy bodies; DRPLA, dentatorubral and pallidoluysian atrophy; FTDP, frontotemporal dementia with parkinsonism; HD Huntington's disease; MJD, Machado Joseph disease; MSA, multiple system atrophy; PD, Parkinson's disease; PS, presenilin; SCA, spinocerebellar ataxis; SOD, superoxide dismutase.
[b]Table adapted from Refs 1 and 44 (with the information regarding Prion taken from Ref. 58, as indicated).

controlled protein degradation in eukaryotes[4]. The basic biochemical steps of the pathway have been reviewed in detail (Fig. 1)[5–7]. Interestingly, some proteins that are linked to neurodegenerative diseases might also be connected to the ubiquitin system (Table 2). For instance, genomic studies in familial PD have discovered mutations in two ubiquitin-related proteins – the ubiquitin-like domain containing protein parkin, and the ubiquitin C-terminal hydrolase L1 (UchL1), which might cause PD (Refs 2,3 and discussed later in this review). In addition, different ubiquitin-like proteins have been found to interact with proteins that cause neurodegenerative diseases (Table 2); thus, the UPP might play secondary roles in these disorders.

Parkinson's disease and the UPP

PD is a prototypical neurodegenerative disease with prominent intracytoplasmic inclusions of proteinaceous material called Lewy bodies. Lewy bodies are one of the defining pathological hallmarks of PD and DLB, and are composed of eosinophilic intracellular neuronal proteinaceous inclusions that mainly contain lipids, neurofilament and related proteins, α-synuclein, synphilin-1, ubiquitin and the ubiquitin-pathway-related enzymes[8–12]. PD is currently the only neurodegenerative disease that is caused by mutations in proteins within the UPP. Thus, understanding how these mutations cause PD could lead to greater insight into other neurodegenerative disorders.

Great advances in our understanding of the etiology of PD have occurred over the past few years[13,14]. Genetic linkage studies have identified several mutations that cause familial PD. Two familial-associated PD genes are part of the UPP: UchL1 (Ref. 3) and parkin, which has a ubiquitin-like domain[2]. Mutations in α-synuclein, one of the major components of the Lewy bodies[15], is also linked to familial PD (Ref. 16). Other families with hereditary PD have been reported but the genes associated with these families are not known[17].

A mutation in UchL1 (Ile93Met) was identified in a small German pedigree composed of two affected family members[3]. UchL1 is one of the most abundant proteins in the brain and belongs to a family of enzymes that is responsible for degrading polyubiquitin chains back to the ubiquitin monomer[18,19]. UchL1 is present in Lewy bodies[20]. The mutation (Ile93Met) was found to decrease the enzymatic activity of UchL1, but how this is linked to PD is not known[3]. Mutations in UchL1 are rare in PD – only two affected

family members in one family have been identified in a large, genome-wide search[21]. The rarity of the mutation suggests that it is either a very rare cause of PD or it is a chance occurrence that is unrelated to the cause of PD in this family.

However, polymorphisms in UchLI might protect against PD, providing potential support for the importance of UchL1 in the pathogenesis of PD (Ref. 22). A malfunction of UchL1 in degrading the polyubiquitin chain could impair the overall efficiency of the ubiquitin system and ultimately increase the accumulation of damaged protein, thus threatening the survival of neurons under any additional unfavorable conditions. An in-frame deletion including exons 7 and 8 of UchL1 in mice causes gracile axonal dystrophy (Gad)[23]. Gad mice have sensory ataxia at an early age, followed by motor ataxia as they age. There is also an accumulation of β-amyloid (Aβ) and ubiquitin deposits, suggesting that the altered function of the de-ubiquitinating system is directly responsible for neurodegeneration.

Parkin belongs to a family of proteins with conserved ubiquitin-like and RING finger motifs[2,24]. Mutations in parkin cause autosomal recessive PD (AR-PD). In the limited neuropathological studies of patients with parkin mutations, there is a selective loss of dopaminergic neurons without the presence of Lewy bodies. *In situ* hybridization studies show that parkin, UchL1 and α-synuclein mRNA have similar expression patterns[25]. Parkin can interact with actin filaments, but how this is related to the pathogenesis of AR-PD is not known[26]. Recently, parkin was reported to function as a ubiquitin E3 protein ligase (Fig. 2)[27–29]. It appears to use both UbcH7 and UbcH8 as its E2 and also utilizes the ER-associated E2s, Ubc6 and Ubc7 (Ref. 30). Familial-associated mutations in parkin have impaired binding to either UbcH7 or UbcH8 and are defective in E3 ubiquitin–protein-ligase activity, which suggests that the disruption of the E3 ubiquitin–protein-ligase activity of parkin is probably the cause of AR-PD (Refs 27–29). Several potential substrates for parkin have recently been identified, one of which is cell division control-related protein 1 (CDCrel-1) (mutations in parkin impair its ability to regulate the turnover of CDCrel-1)[29]. CDCrel-1 belongs to a family of septin GTPases; it has been suggested that it regulates synaptic vesicle release in the nervous system[31]. Whether CDCrel-1 is involved in the release of dopamine (DA) is not yet known but it is possible that mutations in parkin affect CDCrel-1-mediated dopamine release, which ultimately contributes to the Parkinsonian state of AR-PD patients[29]. Because septins are highly conserved proteins[32], it is conceivable that parkin could interact with other septins to regulate their levels as well. Whether CDCrel-1 or a closely related septin accumulates in PD and/or contributes to the pathogenesis of AR-PD, awaits further study.

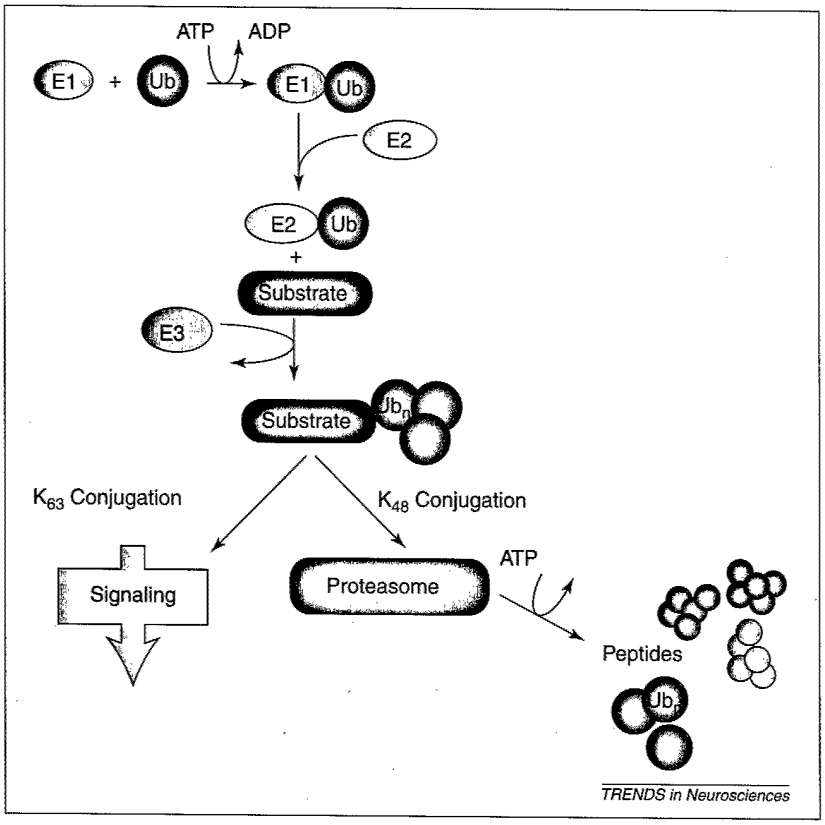

Figure 1. The steps and components in the ubiquitination of substrate proteins in the ubiquitin-proteasomal pathway

Ubiquitin (Ub) is first activated by ubiquitin-activating enzyme (E1) in the presence of ATP. Next, the activated ubiquitin is transferred to ubiquitin-conjugating enzyme (E2). The E2 in conjunction with ubiquitin-protein ligase (E3) recognizes the substrate and mediates the attachment of polyubiquitin chains to the substrate. The polyubiquitin chain is then recognized by the proteasome and degraded in an ATP-dependent manner. E3 is therefore one of the most important factors in the regulation and selectivity of substrates targeted for degradation. There are several established families of E3 enzymes. One of the best known is the HECT domain E3 family, which was first characterized by its representative member, the E6 associated protein (E6-AP)[5]. Another emerging major class of E3 ligases are proteins that contain a RING finger domain[66]. Proteins that are polyubiquitinated by the ubiquitin system are typically targets for degradation by the proteasome[5,6]. However, ubiquitination of protein substrates without proteolysis has recently been described in several systems, thus unveiling potential new regulatory functions for ubiquitin[67–70]. Interestingly, RING-finger E3 ligases seem to play a prominent role in the functional modification of target proteins through ubiquitination in a protein-degradation-independent fashion[67–70]. Some of these functional alterations in target proteins occur through alternative types of ubiquitination, such as lysine $(K)_{63}$ chains[69,70] rather than the more common lysine $(K)_{48}$ ubiquitin chain, which is well known to promote the corresponding proteasomal dependent protein degradation[69,71]. Thus, derangements in the ubiquitin system could potentially lead to alterations in processes that are unrelated to protein degradation. These non-degradative pathways might be attractive drug targets. Abbreviation: Ub_n, polyubiquitin chain.

Parkin is upregulated by unfolded protein stress and has been found to suppress unfolded protein-stress-induced toxicity[28]. The unfolded protein response regulates a variety of proteins, including multiple ER and secretory pathway genes [including proteins involved with ER-associated protein degradation (ERAD)]. Many of the ERAD proteins are components of the UPP. Misfolded proteins are retrotranslocated across the ER membrane into the cytosol, where they are degraded through the ERAD system[33,34]. Because parkin is localized to the microsomal fractions, as well as to the cytosol and Golgi fractions[35], it is conceivable that it is involved in ERAD. Thus, mutations

Table 2. Proteins in the ubiquitin pathway that are connected to neurodegenerative diseases[a]

Disease	UPP proteins potentially linked to neurodegenerative diseases	Function in the ubiquitin pathway	Mutations cause disease?	Disease-related interactors	Refs
AD	Ubiquilin	Linker for E3 ligase and proteasome?	No	PS1, PS2	59
DRPLA	AIP2, AIP4, AIP5	E3 ligase?	No	Atrophin-1	60
HD	hE2-25K	E2	No	Huntingtin	61
PD	Parkin	E3 ligase	Yes	CDCrel-1 Synphilin-1 Pael-R α-Sp22	2,27, 28,30, 37,38
	UchL1	De-ubiquitination enzyme	?	?	3
SCA1	A1Up	Linker for E3 ligase and proteasome?	No	Ataxin 1	62
SCA3/MJD	HHR23A, HHR23B	Inhibits multi-ubiquitin chain formation	No	Ataxin 3	63,64

[a]Parkin functions as an E3 ligase and has four potential substrates: CDCrel-1, synphilin-1, Pael-R and α-Sp22 (see text for full discussion). Atrophin-1, the DRPLA gene product, interacts with a family of WW and HECT domain-containing proteins (AIP2, AIP4, AIP5), which are highly homologous to the HECT domain E3 ligase[60]. HD interacts with the E2 conjugating enzyme hE2-25K (Ref. 61). In SCA1, ataxin-1 interacts with a novel protein, A1Up, which contains both ubiquitin-like (UBL) or ubiquitin associated (UBA) domains[62]. Ataxin-3, the Machado Joseph Disease gene product, interacts with HHR23A and HHR23B, which are homologous to the yeast protein, RAD23 (Ref. 63). Ubiquilin (hPLIC-1), which also has a UBL and UBA domain, interacts with presenilin 1 and 2 (Ref. 59). Mutations in presenilins (PS) 1 and 2 are linked to early-onset familial AD (Ref. 65). Interestingly, A1Up and ubiquilin belong to a highly conserved family of proteins containing UBL and UBA domains. UBL- and UBA-containing proteins are thought to have important functions in protein degradation and possibly link the ubiquitin tagging system with the proteasome. Whether various disease-causing mutations in these neurodegenerative-disease-associated proteins could interfere with the processing of proteins via the UPP (owing to alterations in their binding or disruption of the function of these UBL- and UBA-domain-containing proteins) awaits further study. Abbreviations: A1Up, ataxin-1 interacting protein; AIP, atrophin-1 interacting protein; AD, Alzheimer's disease; CDCrel-1, cell division control-related protein1; DRPLA, dentatorubral and pallidoluysian atrophy; PD, Parkinson's disease.

or deletions of the *parkin* gene could result in accumulation of misfolded substrate proteins in the ER, leading to dopamine cell death in AR-PD (Ref. 28).

Recently, an unfolded putative G-protein-coupled transmembrane receptor – the parkin-associated endothelin-receptor-like receptor (Pael-R) – was found to be a parkin substrate[30]. When overexpressed, Pael-R tends to become unfolded, insoluble and ubiquitinated, and causes unfolded protein-induced cell death[30]. Co-expression of parkin results in protection against Pael-R-induced cell toxicity[30]. Insoluble forms of Pael-R accumulate in the brains of AR-PD patients[30]. Thus, accumulation of Pael-R caused by parkin mutations could result in the selective neurodegeneration of AR-PD. Although Pael-R is a potentially important disease-causing substrate of parkin, its localization in oligodendrocytes and its enrichment both in DA neurons and in other neurons does not entirely explain the selective loss of DA neurons in AR-PD patients[30].

UPP and Lewy bodies

The prominence of ubiquitinated protein species within the Lewy body, and the observation that parkin functions as an E3 ligase, make it conceivable that proteins contained within Lewy bodies are targets of parkin-mediated ubiquitination. Furthermore, the absence of Lewy bodies in patients with parkin mutations suggests that parkin might be involved in the formation of Lewy bodies[29]. Two mutations in α-synuclein – A53T and A30P – cause an early-onset, autosomal dominant form of familial PD (Refs 16,36). In addition, α-synuclein is a major component of Lewy bodies, suggesting that it might play a prominent role in sporadic PD (Ref. 15).

It has been suggested that the ubiquitin system tags α-synuclein for proteasomal degradation. Under adverse conditions, the ubiquitin system might not be able to cope with the rate of formation of damaged and/or mutant α-synuclein, and, thus, ubiquitin- and α-synuclein-positive protein inclusions (i.e. Lewy bodies) would be formed. Using immunological methods in normal human brain, Shimura and colleagues identified a protein complex containing parkin, UbcH7 and a novel glycosylated form of α-synuclein (α-Sp22)[37]. Familial-associated parkin mutants failed to bind α-Sp22 and, in an *in vitro* ubiquitination assay, α-Sp22 was ubiquitinated by normal but not mutant parkin. Interestingly, α-Sp22 accumulated as a non-ubiquitinated form in AR-PD brains. Non-glycosylated

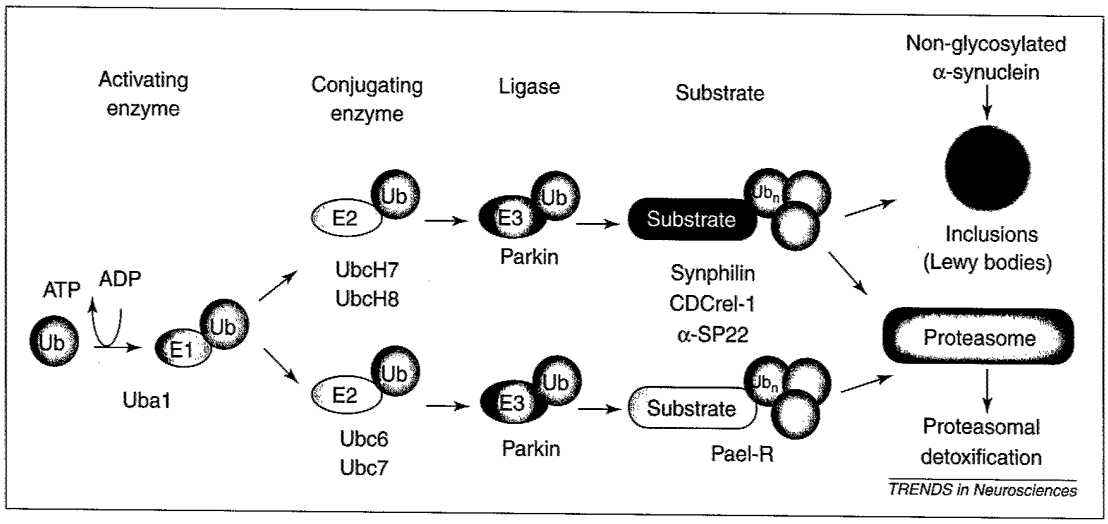

Figure 2. Parkin functions in the ubiquitin-proteasomal pathway as an E3 ubiquitin–protein-ligase

Parkin appears to work in conjunction with the E1 ubiquitin-activating (Uba) 1 and the E2 ubiquitin-conjugating (Ubc) enzymes UbcH7 or UbcH8 to ubiquitinate the target proteins CDCrel-1, α-Sp22 or the α-synuclein interacting protein, synphilin-1. Ubiquitination of CDCrel-1 by parkin enhances its degradation via the proteasomal degradation system[29]. Ubiquitination of synphilin-1 in the presence of non-glycosylated α-synuclein by parkin leads to ubiquitinated cytoplasmic Lewy-body-like inclusions. α-Sp22 accumulates in the brains of patients with Parkinson's disease (PD) owing to familial associated parkin mutations. Whether α-Sp22 accumulates in Lewy bodies is not known. Parkin also appears to participate in endoplasmic-reticulum-associated protein degradation (ERAD) (see text for discussion) and utilizes the ER-associated E2s, Ubc6 and Ubc7 to degrade toxic unfolded proteins such as the parkin-associated endothelin-receptor-like receptor (Pael-R). Pael-R accumulates in autosomal recessive PD (AR-PD) brains and cell death induced by Pael-R overexpression is rescued by parkin. Abbreviations: CDCrel-1, cell division control-related protein 1; Ub_n, polyubiquitin chain.

α-synuclein, the major species in brain, does not appear to be a parkin substrate: both *in vitro* (in heterologous transfection assays in cell lines) and in brain[30,37,38], parkin fails to interact with non-glycosylated α-synuclein, and fails to ubiquitinate non-glycosylated α-synuclein[38]. Thus, parkin and α-synuclein might be linked in a common pathogenic mechanism through glycosylation of α-synuclein, and this interaction could result in ubiquitinated α-synuclein in PD.

Although α-Sp22 appears to be ubiquitinated by parkin *in vitro*, as yet no ubiquitin-positive α-synuclein species have been definitively isolated *in vivo*. Immunohistochemical studies show that Lewy bodies have a distinct central ubiquitin-positive domain, whereas α-synuclein-positive staining primarily occurs in the peripheral and outer domain of the Lewy body[8,10], suggesting that these two proteins might not be in the same compartment. In addition, pale bodies (or diffuse 'cloud-like' inclusions) are found in PD and DLB that are only α-synuclein positive[8], suggesting that non-ubiquitinated α-synuclein protein inclusions do exist. Thus, the issues of whether α-synuclein is being ubiquitinated *in vivo*, and how proteins contained within Lewy bodies are ubiquitinated, remain to be resolved. A potential clue to this process comes from recent studies in which parkin was shown to interact with, and ubiquitinate, the α-synuclein-interacting protein, synphilin-1[38–41]. *In vitro* reconstitution assays indicate that synphilin-1 is a direct protein target of parkin[38] and is enriched in Lewy bodies[12]. Interestingly, co-transfection of parkin, α-synuclein and synphilin-1 results in the formation of ubiquitin-positive protein inclusion bodies[38], and familial-associated mutations in parkin disrupt the formation of ubiquitin-positive protein inclusions. Thus, parkin and the major non-glycosylated form of α-synuclein might also be linked in a common pathogenic pathway through their interaction with synphilin-1[38], in addition to the direct interaction with glycosylated α-synuclein. Furthermore, parkin and synphilin-1 appear to be required for the formation of ubiquitinated α-synuclein inclusions[38].

Inclusion body: friend or foe?

The UPP appears to be at the intersection of whether a toxic protein is degraded or whether it is packaged into an inclusion. Molecular chaperones also participate in attempts by the cells to suppress aggregate formation. One general hypothesis is that ubiquitinated protein aggregates provide a nucleation center for the formation of inclusion bodies. Aggresomes appear to be part of the general cellular response to the formation of aggregated proteins and it appears that aggregated proteins are delivered specifically to inclusion bodies by dynein-dependent retrograde transport on microtubules[42]. The accumulation of these inclusion bodies might subsequently induce neuronal dysfunction and/or cell death leading to neurodegeneration[43–46]. Neurodegeneration could be induced by the intracellular aggregates overwhelming the capacity of the protein-folding chaperones and/or the UPP to degrade important cellular regulatory factors, leading to a positive feedback

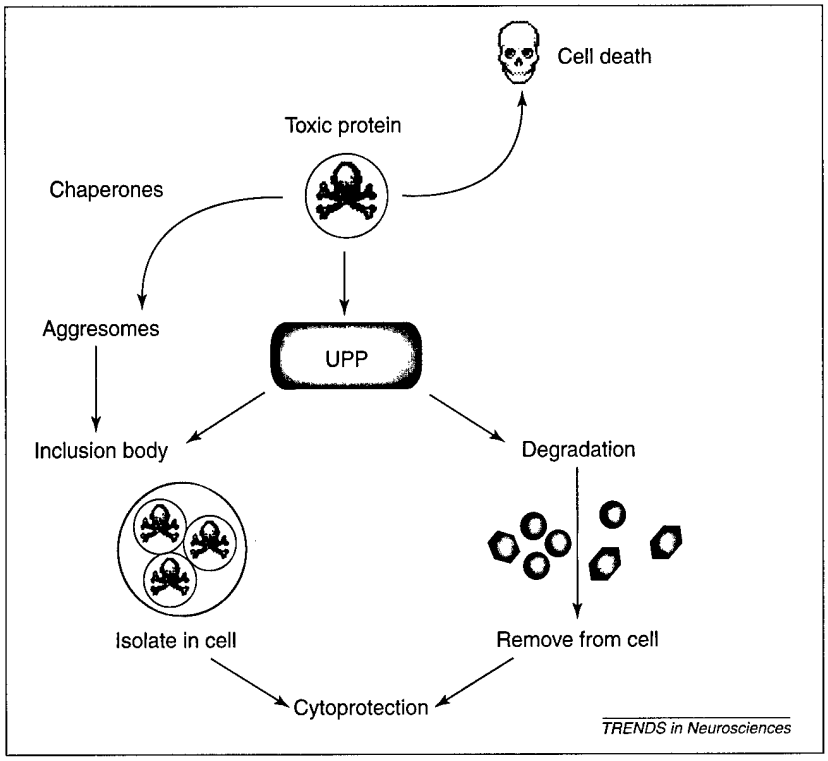

Figure 3. A toxic or damaged protein can be detoxified via at least two pathways

The first involves the ubiquitin-proteasomal pathway (UPP) where the protein is tagged for degradation by the proteasome. If the toxic or damaged protein exceeds the capacity of the proteasome the ubiquitinated protein might serve as a nucleation center for aggregates and inclusion bodies. Aggresomes might be intermediates in the formation of inclusion bodies. Inclusion bodies and the UPP appear to work in a coordinated fashion to protect the cell from toxic or damaged proteins. The capacity of both of these systems might be overwhelmed, leading to further compromise in a feed-forward pathway that ultimately results in the demise of the neuron.

mechanism in which increased aggregation leads to a further decline in the UPP and disruption of fundamental cellular events, and ultimately to neuronal cell death[46].

Although intracellular aggregates might eventually be toxic to cells[46], accumulating evidence suggests that the presence of inclusion bodies is not necessarily deleterious and that, in fact, they might be protective. Thus, the question arises: are inclusion bodies toxic or are they protective agents against some of the more toxic intermediate protein-aggregate species? In PD, for instance, the prefibrillar α-synuclein intermediate might be more toxic than the fibrillized α-synuclein protein aggregates[47,48].

Together with various chaperones, the ubiquitin system might promote the formation of inclusion bodies to render the damaged or mutant protein less toxic than its soluble form. It has been proposed that Lewy bodies are protective in PD (Refs 47,49). As discussed earlier, parkin belongs to a family of proteins with ubiquitin-like domains; this class of proteins might be important in protein folding and degradation, linking the ubiquitin tagging system to the proteasomal apparatus. The finding that parkin is an E3 ligase, and that it can protect cells from unfolded protein stress, further supports this idea. Based on the hypothesis that inclusion bodies might be protective, it is tempting to speculate that parkin could be responsible for detoxifying damaged proteins. One potential pathway for this is through the formation of protein inclusions like Lewy bodies, which could render damaged proteins less toxic. In addition, parkin might tag proteins with polyubiquitin chains for degradation through the proteasome. Both pathways could work together to protect the cell from toxic mutant and/or damaged proteins (Fig. 3). The observations that AR-PD patients develop PD symptoms at an earlier age than other patients, that they accumulate the parkin substrates Pael-R and α-SP22, and that they demonstrate an absence of Lewy bodies appears to support this scenario.

Other studies have suggested that the formation of inclusion bodies is one of the strategies of the cell to process damaged and/or mutated potentially toxic proteins and that, given a chance, the cell will recover from such stress (Fig. 3). In the mouse model of HD, nuclear inclusions (NI) are present in surviving neurons, suggesting that the inclusion body might itself be protective[50]. In the mouse model of SCA1, mutant ataxin-1 that cannot self-aggregate is still toxic to neurons[51]. In the fly model of polyglutamine disease, overexpression of protein chaperones, such as HSP40 or HSP70, protects against polyglutamine-induced toxicity without a visible effect on NI formation[52–54]. In another interesting study, mice with a pathogenic ataxin-1 transgene crossed with mice with a defective ubiquitin system (mutation in E6AP) resulted in enhanced ataxin-1-mediated toxicity, despite decreased formation of ubiquitinated NI (Ref. 55). In an *in vitro* model, conditions that prevent the formation of NI and ubiquitination in neuronal culture were found to enhance mutant huntingtin-induced cell death[56].

In a recent exciting report, lowering the expression of the toxic HD transgene expressing pathogenic expanded polyglutamines in symptomatic mice reversed the neuropathological and behavioral abnormalities, clearly indicating that neurons with inclusion bodies are not those that are dying and that, in fact, inclusion bodies could be reversible protein reservoirs[57]. Despite the notion that inclusions might, in part, be protective, neurons ultimately fail to compensate for the abnormal and/or toxic protein accumulations and die. However, the possibility that inclusions are, in part, protective and reversible means that it could be possible in the future to improve or even reverse neurodegenerative disease before significant neuronal cell death has occurred.

Concluding remarks

It is clear that the UPP is emerging as a major player in neurodegenerative diseases and a full understanding of this intricate system must be achieved to better understand the

pathogenesis of these devastating disorders. Most studies have focused on the biosynthetic anabolic pathways of proteins involved in neurodegenerative diseases. Very little attention has been given to the degradative and catabolic pathways. Greater understanding of these pathways will be required to understand fully the pathogenesis of neurodegenerative disorders. The challenge in the future will be to identify ways to harness the UPP for treatment of neurodegenerative disorders. Inhibition of the UPP might be expected to worsen most neurodegenerative disease. Augmentation of the UPP possesses unique challenges, such as delivery of UPP components to the nervous system or identification of drugs that enhance the degradation of damaged and toxic proteins without compromising normal UPP functions.

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Superoxide dismutase and the death of motoneurons in ALS

Joseph S. Beckman, Alvaro G. Estévez, John P. Crow and Luis Barbeito

Amyotrophic lateral sclerosis (ALS) is a lethal disease that is characterized by the relentless death of motoneurons. Mutations to Cu–Zn superoxide dismutase (SOD), though occurring in just 2–3% of individuals with ALS, remain the only proven cause of the disease. These mutations structurally weaken SOD, which indirectly decreases its affinity for Zn. Zn-deficient SOD induces apoptosis in motoneurons through a mechanism involving peroxynitrite. Importantly, Zn-deficient wild-type SOD is just as toxic as Zn-deficient ALS mutant SOD, suggesting that the loss of Zn from wild-type SOD could be involved in the other 98% of cases of ALS. Zn-deficient SOD could therefore be an important therapeutic target in all forms of ALS.

Remarkably, all voluntary movement depends upon fewer than one million motoneurons localized in the ventral horn of the spinal cord that directly innervate muscles. Amyotrophic lateral sclerosis (ALS) results from the progressive death of these few lower motoneurons, which causes rapid muscle degeneration and paralysis, leaving the victim cognitively intact but unable to interact with the world. ALS also involves the death of large pyramidal neurons in the region of the motor cortex that innervates the lower spinal motoneurons, which reinforces specific reflexes and results in a spastic rather than flaccid paralysis. Excellent general reviews on the pathogenesis in ALS have recently been published[1,2]. This article focuses specifically on how superoxide dismutase (SOD) might be involved in sporadic as well as familial ALS.

SOD mutations in ALS

In 1993, 13 mutations to the cytosolic Cu–Zn SOD were discovered in about 2–3% of individuals with ALS with ~90 different SOD mutations now reported[3]. The vast majority of these mutations are missense point mutations, although a few deletions and insertions have been reported in the C-terminal region (Fig. 1). Although SOD is a small protein of 153 residues, mutations occur at over 40 different locations, which are strategically important for stability of the SOD backbone. However, ~98% of ALS cases do not have mutations in SOD or defects in other antioxidant defense systems, including the mitochondrial manganese SOD and the extracellular Cu–Zn SOD. Because the familial disease is strikingly similar to sporadic ALS, understanding the effects of the SOD mutations might shed light on the biochemical basis of sporadic ALS.

As the chief cell responsible for output from the central nervous system, motoneurons maintain a high metabolic rate and are susceptible to oxidative stress[4]. Molecular oxygen is a strong oxidant and can rob electrons from many biological molecules to form the oxygen radical superoxide ($O_2^{\bullet-}$). However, superoxide is detoxified and maintained at a concentration in the low femtomolar range by intracellular concentrations of SOD that are typically >10 μM (Ref. 5). The SOD mutations in individuals with ALS are dominant, which suggests they confer a toxic gain of function, rather than simply diminishing superoxide-scavenging activity[6]. Strong evidence for the causal role of SOD mutations in ALS is derived from experiments in which transgenic mice that overexpressed specific ALS mutations were shown to develop progressive paralysis caused by the death of motoneurons[7]. Because the complete knockout of

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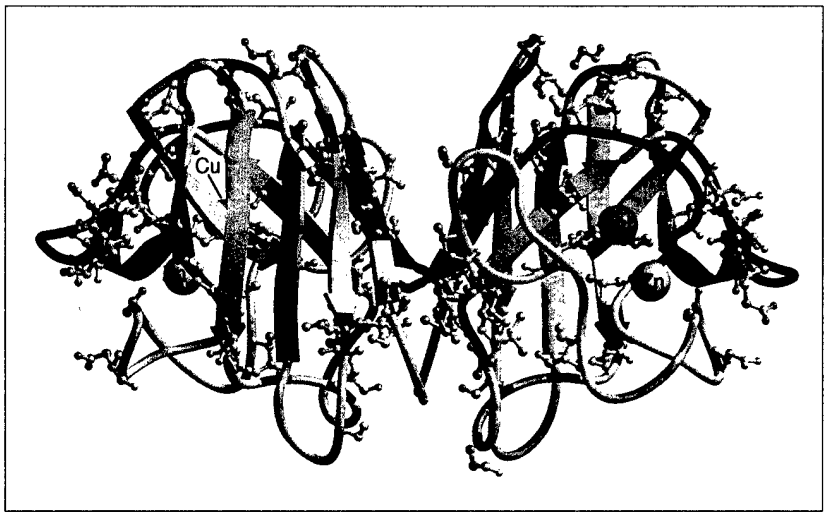

Figure 1. Sites of mutations in Cu–Zn superoxide dismutase

The protein is a homodimer with the active site of the right subunit facing out of the page. The side-chains of mutated amino acids are shown in light blue and on the backbone as dark blue. The majority of the mutations are clustered on the top and bottom of the β barrel, in the dimer interface or along one of the loops that forms part of the Zn-binding pocket of superoxide dismutase (SOD). A major part of the C-terminal region (purple) can be entirely deleted in a few individuals. The only parts of the SOD molecule where mutations have not been found are in the Zn-binding groups and on the residues facing out on backside of the β barrel, opposite the active site.

the endogenous gene encoding SOD in the mouse does not produce motoneuron disease, toxicity cannot be solely caused by the loss of superoxide scavenging activity[8]. Recent evidence from transgenic mice has established that mutant SODs selectively activate apoptosis in motoneurons[9–11].

What makes SOD toxic?

The apparent gain of function conferred by the SOD mutations in ALS remains elusive. It was originally proposed that the SOD mutants might be more likely to catalyze tyrosine nitration by peroxynitrite[12], but this theory is too simplistic[13]. One important current theory posits that toxicity is independent of oxidative stress and is caused by protein aggregation[14]. The basis for this hypothesis lies in the observation that the toxicity of SODs in ALS appears to be independent of the capacity of the cell to scavenge superoxide; disease progression in transgenic mice is independent of the presence of SOD (the same regardless of whether the endogenous mouse SOD is absent or whether wild-type human SOD is overexpressed sixfold[15]). However, aggregates of SOD protein *per se* have not been demonstrated to be toxic[16]. Metal-deficient apoSODs are nontoxic to motoneurons even though they are considerably more prone to aggregation[13]. Furthermore, mice carrying the A4V SOD mutation (alanine to valine substitution at position 4) do not develop motoneuron disease (though, paradoxically, this mutation causes the most rapid progression of ALS in humans[7]). The level of A4V SOD expression in mice is similar to that of the G93A mutant (glycine to alanine substitution at position 93) and produces large aggregates in motoneurons without cell death[7]. Aggregation of neurofilaments and other proteins is certainly a hallmark of ALS, but aggregation of the SOD protein *per se* might not be the primary cause of toxicity. Indeed, nitrative stress generated by SOD and peroxynitrite could promote the aggregation of structural proteins.

Transgenic SOD mice provide vital clues into the pathogenesis of ALS, but deciphering the mechanisms responsible for SOD toxicity is nearly impossible without understanding the biochemical properties of the protein. In an attempt to understand how SOD causes ALS, the redox chemistry of the purified proteins with known metal contents has been studied and their toxicity characterized in isolated motoneurons[13]. An important result of this work is that most of the ALS mutant proteins can correctly fold to produce fully functional SODs that are equally protective to isolated motoneurons as wild-type SOD (Ref. 13). This might explain how some people can express ALS mutant SODs for as long as 80 years before the symptoms of ALS appear.

However, the mutant SODs do have more problems binding metals[17]. The ALS mutants have diminished Zn affinity and often accumulate in Zn-deficient states when expressed *in vitro*[18]. The reduced affinity for Zn appears to be an indirect consequence of the ALS mutations structurally weakening the backbone of the SOD protein[19]. Because Zn is held about 7000-fold less tightly than Cu even in wild-type SOD, structural defects cause Zn to be lost before Cu (Ref. 18).

Oxidant production by Zn-deficient SOD

The loss of Zn profoundly alters the redox properties of SOD and makes SOD toxic to motoneurons[13]. Rather than acting as a scavenger of superoxide, Zn-deficient SOD can steal electrons from cellular antioxidants and transfer these electrons to oxygen to produce superoxide (Fig. 2). One can see this chemistry as it occurs: the loss of Zn changes the color of SOD from green to blue – direct evidence of the altered environment of the enzyme-bound Cu. When ascorbate is added to the blue-colored Zn-deficient SOD, the protein quickly becomes colorless as the copper (Cu^{2+}) is reduced. The blue color gradually reappears as Cu^{+} gives up its electron to oxygen, thereby producing superoxide.

This slow production of superoxide by Zn-deficient SOD is not toxic by itself because remaining Cu–Zn SOD present in a cell recaptures superoxide. However, nitric oxide reacts so rapidly with superoxide to produce peroxynitrite that it can effectively compete with SOD for

superoxide. Nitric oxide is small enough that it might even be reacting with oxygen transiently bound in the active site of reduced SOD to drive the formation of peroxynitrite (Fig. 2). Furthermore, wild-type Cu–Zn SOD does not prevent peroxynitrite formation[13], in part because it also can form peroxynitrite when reduced (Fig. 2). This *in vitro* finding provides a possible explanation for the failure of overexpressed wild-type SOD to prevent the toxicity of ALS mutants in transgenic mice and a basis for the dominant inheritance of SOD mutations in humans.

The role of peroxynitrite in motoneuron death

In order to understand more fully the toxicity of SOD and nitric oxide, we have used cultured motoneurons, one of only two neuronal types that can be isolated at high purity. When grown in the presence of any of several different neurotrophic factors, motoneurons isolated from spinal cords of embryonic rats develop the phenotype of a mature motoneuron over a period of about one week[20]. If these trophic factors are withdrawn at any point, the motoneurons undergo apoptosis[21]. Cell death requires the simultaneous production of both nitric oxide and superoxide, implicating peroxynitrite as an early intermediate for activating apoptosis in trophic factor-deprived motoneurons[20]. Before motoneurons undergo apoptosis, they induce neuronal nitric oxide synthase and become immunoreactive for nitrotyrosine, a marker of peroxynitrite. Inhibiting nitric oxide synthesis prevents apoptosis, protection that is lost when physiologically realistic concentrations (<100 nM) of nitric oxide are generated exogenously. To test for the involvement of superoxide, methods have been developed to deliver SOD intracellularly to motoneurons using liposomes. Intracellular delivery of Cu–Zn SOD protected motor neurons as well as inhibitors of nitric oxide synthesis[21].

Zn-deficient SOD is toxic to motoneurons

The ability to deliver SOD entrapped in liposomes to motoneurons has enabled the direct testing of whether Zn-deficient SOD is toxic to motoneurons[21]. Four different ALS mutants and wild-type SOD were prepared either replete with Cu and Zn, or depleted of Zn (Ref. 13). Delivery of wild-type Cu–Zn SOD or any of the four ALS mutant SODs replete with Cu and Zn protected motoneurons equally well from trophic factor deprivation. However, the Zn-deficient forms of ALS mutant and wild-type SOD induced tyrosine nitration and motoneuron apoptosis, even in the presence of trophic factors. Inhibition of nitric oxide synthesis prevented death and blocked accumulation of nitrotyrosine in the motoneurons. Importantly, Zn-deficient wild-type SOD was just as toxic as the mutant SODs. On the basis

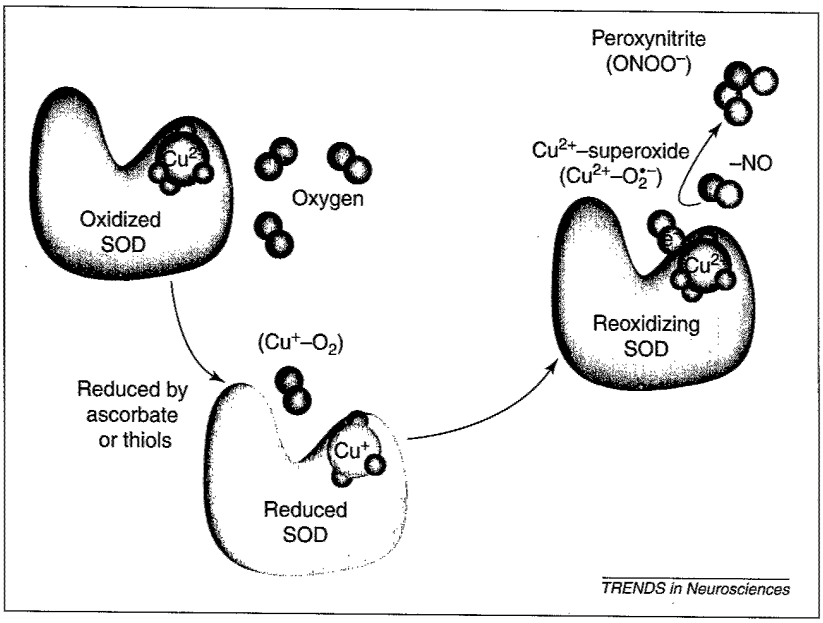

Figure 2. Production of peroxynitrite from oxygen and nitric oxide

Zn-deficient SOD reacts 3000 times faster than Cu–Zn superoxide dismutase (SOD) with ascorbate, leaving the Cu in a reduced state. The reduced Cu in SOD reacts over a period of minutes with oxygen to produce superoxide. Nitric oxide is such a small and highly diffusible molecule that it can enter the active site of SOD to combine with oxygen interacting with the reduced Cu to produce peroxynitrite. Wild-type Cu–Zn SOD does not inhibit this process. Indeed, Cu–Zn SOD can also produce peroxynitrite from oxygen and nitric oxide about as well as Zn-deficient SOD. However, only superoxide can efficiently reduce Cu–Zn SOD.

of these results, it is proposed that the mutations to SOD do not directly confer the gain of function, rather they increase the susceptibility to lose Zn (with Zn-deficient SOD being responsible for the death of motoneurons). Thus, wild-type SOD can participate in sporadic ALS if it becomes Zn deficient.

The role of Cu in ALS

Cu is essential for the toxicity of Zn-deficient SOD and apoSOD (SOD lacking in Cu and Zn) itself is not toxic to the motoneurons[13]. Recently, considerable attention has focused on the Cu chaperone protein for SOD (CCS). In knockout mice that lack CCS, the activity of endogenous SOD is decreased by about 70–80% (Ref. 22). Although CCS is the major source of Cu *in vivo*, SOD can apparently acquire Cu from other sources in cells as 20–30% of activity remains in the knockout mice. Cu is absolutely required for SOD to scavenge superoxide[23]. A probable source for SOD to acquire Cu is the mitochondria, where a separate Cu transport system provides substantial amounts of Cu for cytochrome-c oxidase. The alternative sources of Cu might explain why deletion of the gene encoding CCS has little effect on the development of motoneuron disease in ALS mutant SOD mice[14]. Other studies have shown that CCS selectively inserts Cu into Zn-containing SOD (Ref. 24), which will produce Cu–Zn

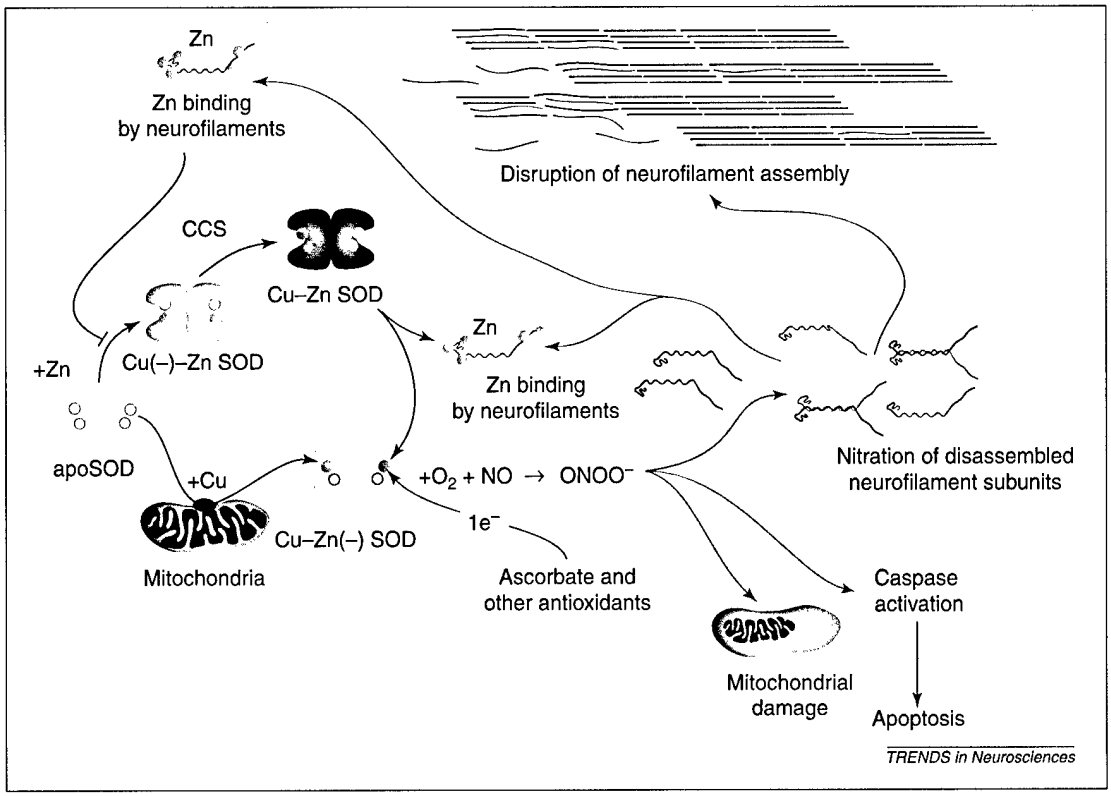

Figure 3. Proposed interactions of superoxide dismutase and neurofilaments leading to the death of motoneurons

Newly synthesized superoxide dismutase (SOD) apparently binds Zn before the Cu chaperone for SOD (CCS) inserts Cu to form protective Cu–Zn SOD. However, the apo-protein is a strong metal chelator and might pick up Cu adventitiously from the mitochondria or from metallothionine. Zn-deficient SOD can produce peroxynitrite from nitric oxide, oxygen, and electrons taken from ascorbate and other intracellular antioxidants. The disassembled subunits of neurofilaments are particularly susceptible to nitration and oxidation by peroxynitrite. These damaged subunits can disrupt the structure of neurofilaments and the accumulation of neurofilaments forms a major new pool to bind Zn. This can accelerate the accumulation of Zn-deficient SOD by either withdrawing Zn from Cu–Zn SOD or blocking the incorporation of Zn into the apo-protein. As more Zn-deficient SOD accumulates, the additional formation of peroxynitrite can damage mitochondria and eventually drive motoneurons to apoptosis.

SOD. When replete with Zn, even SODs that are mutated in ALS are protective. In the absence of the CCS protein, the mutant SODs might acquire Cu without Zn from other sources (e.g. the mitochondria) contributing to the formation of Zn-deficient SOD (Fig. 3). This could explain why mitochondrial damage is the earliest marker of injury in ALS–SOD mutant mice[25].

Nitric oxide and peroxynitrite in ALS

There has been considerable skepticism about the role of nitric oxide and peroxynitrite in ALS (Ref. 14). One major objection is the failure of the neuronal NOS knockout to provide any protection against ALS or mutated SOD in mice[26]. However, the knockout for neuronal NOS is incomplete, leaving the β-splice-variant expressed in skeletal muscle and some neuronal tissues. Levels of the β variant are increased in reactive astrocytes in ALS transgenic mice[27]. Motoneurons also express endothelial nitric oxide synthase[28] and inducible nitric oxide synthase is upregulated in ALS (Refs 29,30). Inhibition of NOS has been shown to protect motoneurons from degeneration after ventral root avulsion[31] and in wobbler mice[32].

A second line of evidence that supports a role for nitric oxide and peroxynitrite in ALS, is the finding by multiple laboratories that there is increased nitrotyrosine in lower motoneurons of transgenic mice, and in both upper and lower motoneurons in individuals with ALS (Refs 30,33–35). Strong *et al.* have isolated nitrated neurofilaments from individuals with ALS, but similar levels of nitration are observed in neurofilaments isolated from spinal cords of control subjects[36]. Because motoneurons constitute only a miniscule fraction of spinal cord, contributing less than about 1% of total protein, any quantitative differences would be lost. Furthermore, nitration is not specific for ALS and might have significant functional consequences for neurofilament proteins in other neurological diseases.

Nitration of neurofilaments

In cultured motoneurons, Zn-deficient SOD can generate enough peroxynitrite to activate apoptosis, but other

neuroprotective mechanisms *in vivo* will resist the loss of these neurons. However, the continued generation of sub-lethal concentrations of peroxynitrite has other actions that might amplify injury until the threshold for apoptosis is crossed. For example, the structural proteins that form neurofilaments are particularly susceptible to tyrosine nitration by peroxynitrite. Because of their long axons, motoneurons contain enormous quantities of neurofilament proteins. When disassembled, several tyrosines in the coiled-coil domain of neurofilament subunits are exceptionally vulnerable to nitration[37]. Strong *et al.* have shown that the disassembled neurofilament light (L) subunits from spinal cords of individuals with ALS are more intensely nitrated than assembled neurofilaments[36]. A small fraction of nitrated subunits can disrupt the assembly of non-nitrated neurofilaments and might contribute to the aberrant conglomerates of neurofilaments found in the soma and proximal axons in motoneurons degenerating in ALS. Peroxynitrite damage to structural proteins also appears to be involved in Parkinson's disease. Peroxynitrite induces aggregation of α-synuclein *in vitro* and nitrated α-synuclein is found in the core of Lewy bodies[38].

Zn deficiency in motoneurons

A major gap in our knowledge is what causes SOD to become Zn deficient in motoneurons. Although Zn is bound to hundreds of different proteins, the concentration of free Zn in cells might be less than one atom per cell[39]. Curiously, neurofilaments, the predominant protein expressed in motoneurons, also have enormous capacities for binding Zn and can outcompete SOD for binding Zn *in vitro*[18]. The high concentration of neurofilament proteins in motoneurons, combined with their high Zn-binding capacity, could explain the specificity of SOD for killing motoneurons in ALS. Deletion of the neurofilament L subunit protects motoneurons against ALS SOD in transgenic mice[2]. The interactions between neurofilaments and Zn-deficient SOD can potentially create a vicious cycle leading to the death of motoneurons (Fig. 3). As more Zn is bound to neurofilaments, more Zn-deficient SOD accumulates and generates more peroxynitrite, which nitrates neurofilaments, causing more neurofilaments to aggregate until enough peroxynitrite is generated to activate apoptosis (Fig. 3). Aberrant upregulation of neurofilament proteins or other Zn-binding proteins such as metallothionein could result in the production of Zn-deficient SOD from even the wild-type enzyme and could, thus, underlie the genesis of sporadic ALS.

Concluding remarks

Although controversial[40], the Zn-deficient SOD hypothesis offers a rational mechanism to explain how so many different mutations could have the same effect, and how wild-type SOD can participate in sporadic ALS. It predicts that disruption of Zn metabolism in motoneurons is important in the origin of both sporadic and familial ALS. Zn-deficient SOD offers a target suitable for high-throughput screening to develop new therapeutic agents. One crucial prediction is that any such agent should work as well as, or possibly better, in sporadic ALS than in the much rarer familial SOD ALS cases.

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Note added in proof

A new paper that supports our suggestion of Cu uptake in the mitochondria by SOD has recently been accepted for publication. Sturz, L.A. *et al.* A fraction of yeast Cu–Zn superoxide dismutase and its metallochaperone, CCS, localize to the intermembrane space of mitochondria: a physiological role of SOD1 in guarding against mitochondrial oxidative damage. *J. Biol. Chem.* (in press).

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Molecular mechanisms of brain aging and neurodegenerative disorders: lessons from dietary restriction

Tomas A. Prolla and Mark P. Mattson

The application of modern molecular and cell biology technologies to studies of the neurobiology of aging provides a window on the molecular substrates of successful brain aging and neurodegenerative disorders. Aging is associated with increased oxidative stress, disturbances in energy metabolism and inflammation-like processes. Dietary restriction (DR) can extend lifespan and might increase the resistance of the nervous system to age-related neurodegenerative disorders. The neuroprotective effect of DR involves a preconditioning response in which the production of neurotrophic factors and protein chaperones is increased resulting in protection against oxyradical production, stabilization of cellular calcium homeostasis, and inhibition of apoptosis. DR might also enhance neurogenesis, synaptic plasticity and self-repair mechanisms.

The extension of the maximum lifespan of rodents by dietary restriction (DR) was shown 65 years ago[1]. When started early in life or in middle age, DR increases both mean and maximum lifespan, reduces and delays both spontaneous and induced cancers, and lowers the incidence of several other age-related diseases[2]. Indeed, aged rodents maintained on DR appear younger than their freely eating counterparts (Fig. 1). The effects of DR on average and maximum lifespan and mortality rate in rodents strongly support the view that DR slows fundamental aspects of the aging process. This hypothesis is also supported by the fact that DR can retard the aging process in diverse species, such as *Tokophyra* (a protozoan), *Daphnia* (the water flea) and *Lebistes* (the guppy)[2]. Despite intensive investigation, the mechanism(s) by which DR retards aging remains unknown, in part because animals on DR display physiological changes that fit many current aging theories. Indeed, DR reduces not only O_2 consumption of the whole animal but also thyroid hormone levels and body temperature, suggesting a lower metabolic rate[2]. DR also reduces blood glucose levels, increases insulin sensitivity and preserves certain immunologic functions[2]. Given this plethora of effects, it is difficult to distinguish primary from secondary effects of DR on aging retardation. Although extension of lifespan has not been definitively established in humans or other primates, ongoing trials in rhesus monkeys indicate that some physiological effects associated with DR in rodents, such as decreased blood glucose, reduced insulin levels, improved insulin sensitivity and lowering of body temperature, also occur in monkeys[3,4].

A theory that is gaining favor is that DR induces a global metabolic response that results in higher metabolic efficiency, lower production of toxic metabolic byproducts and the induction of specific adaptative responses to stress[5–8]. Global stress adaptations, such as that mediated by the oxyR regulon, are well characterized in bacteria[9] and probably exist in mammals. Other evidence for the role of stress responses in longevity comes from recent work in *Drosophila melanogaster* and *Caenorhabditis elegans* that establish that aging rates can be determined genetically. Specifically, aging in *D. melanogaster* is significantly retarded

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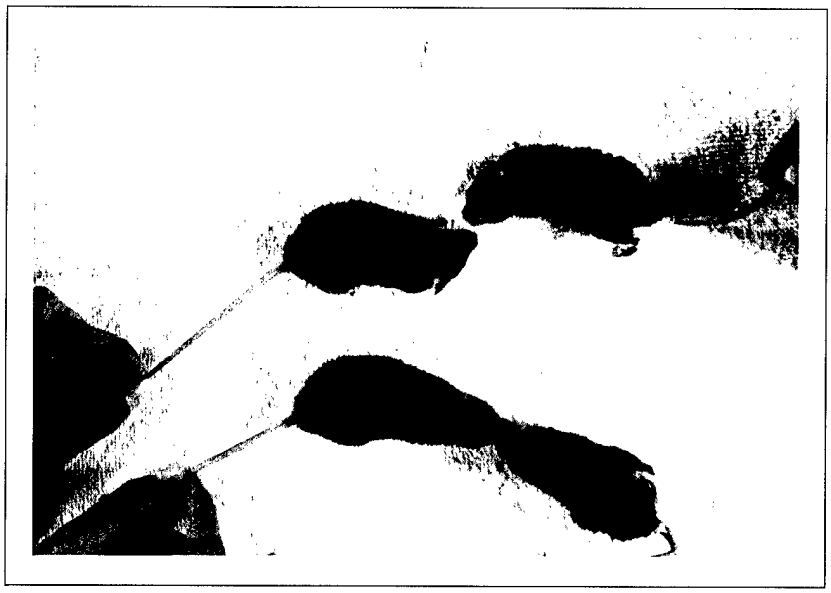

Figure 1. Thirty-two-month-old mice maintained on either dietary restriction or a control diet

Mice on dietary restriction (DR; left) show several signs of retarded aging, including extended lifespan and the delayed development of age-related diseases, compared with mice on a control diet (right). Photograph courtesy of Richard Weindruch.

by increasing the levels of antioxidant enzymes[10] or by genetic selection for either long-lived strains[11] or strains with high resistance to oxidative stress[12]. Based on these experiments, it appears that resistance to oxidative and other stresses leads to pleiotrophic effects, which include aging retardation. Evidence linking metabolic control to aging derives from the demonstration that mutations in the insulin-related transcription factor DAF-16 in *C. elegans*[13], or the gene encoding the insulin-like receptor in *D. melanogaster*, control lifespan[14]. Interestingly, mutations in DAF-2 are also associated with increased resistance to thermal exposure and oxidative stress[15].

The aging brain

Recent studies indicate that in normal brains aging is associated with subtle morphological and functional alterations in specific neuronal circuits, rather than large-scale loss of neurons[16] (Fig. 2). In fact, aging of the central nervous system (CNS) in diverse mammalian species shares many common features, such as dendritic regression in pyramidal neurons, synaptic atrophy, decrease of striatal dopamine receptors, accumulation of fluorescent pigments, cytoskeletal abnormalities and reactive astrocytes and microglia[17]. Although age-associated defects in particular neuronal circuits have been described, the molecular basis of aging in the brain remains unknown. Postulated mechanisms include instability of nuclear and mitochondrial genomes that leads to alterations in gene expression, production of reactive oxygen species (ROS) that damage crucial targets[6], neuroendocrine dysfunction associated with exposure to corticosteroids[18] and altered calcium metabolism[19]. In rodents and humans without clinical neurological disease, brain aging is associated with a general increased incidence of activated astrocytes and microglia. Glial fibrillary acidic protein (GFAP), an intermediate filament that mediates the extension of astrocyte processes, is a widely used marker for astrocyte activation. The amount of mRNA encoding GFAP increases during aging, as determined by *in situ* hybridization[20], but there are considerable differences between brain regions, with the most prominent increases in hippocampal molecular layers that receive major inputs from the neocortex. In myelinated fibers of the corpus callosum, both astrocytes and microglia are activated in middle-aged rats[20]. Importantly, once activated, microglia can produce several pro-inflammatory mediators including cytokines [e.g. interleukin (IL)-1, IL-6 and tumor necrosis factor α], cytotoxic complement components, ROS, nitric oxide and excitotoxins such as quinolinic acid[21]. Inflammatory reactions are not limited to glial cells, as neurons can also exhibit induction of inflammatory factors, such as complement C1q (Ref. 22). There is now solid support for the hypothesis that the induction of a neuroinflammatory cascade contributes to many age-related neurodegenerative disorders.

Molecular profile of the aging brain in mice

Normal aging is associated with specific transcriptional profiles

To search for mechanisms of aging in the brain, DNA microarray analysis was recently performed on the neocortex and cerebellum of young (5-month-old) and aged (30-month-old) mice[23]. Of the 6347 genes surveyed, the expression of only 67 (1%) increased by more than 1.7-fold with aging in the neocortex, whereas the expression of 63 (1%) increased more than 2.1-fold with aging in the cerebellum. Alterations in mRNA levels, as detected by DNA microarray analysis, could represent changes in transcription, mRNA stability or turnover (a complete listing of age-related changes in gene expression is available at http://www.wisc.edu/genetics/CATG/prolla/data/aging/index.html). Of these, 20% (14 out of 67) and 27% (17 out of 63) can be assigned to an immune or inflammatory response in the neocortex and cerebellum, respectively. Several genes in this category, including those encoding microglial and macrophage migration factors, intracellular adhesion molecule 2, Exodus-2 and MPS1, and the CD40L receptor involved in lymphocyte activation, were common to the two brain regions, although fold-changes tended to be higher in the cerebellum. Interestingly, there was a concerted induction of C4, C1qA, C1qB and C1qC

components of the complement cascade, a part of the humoral immune system involved in inflammation and cytolysis. Production of complement proteins in the brain and subsequent generation of proinflammatory peptide fragments could contribute to the neuronal damage associated with stroke[24] and Alzheimer's disease[25] (AD). Consistent with a state of oxidative stress and accumulation of damaged proteins, genes involved in a stress response accounted for 24% (16 out of 67) and 13% (8 out of 63) of those induced in the neocortex and cerebellum, respectively, in 30-month-old animals. These included the heat-shock factors Hsp40, α-β-crystallin, Hsp27, Hsp59 and Hsc70. A possible molecular basis for the induction of chaperones and lysosomal proteases in the aged brain is protein oxidation subsequent to inflammatory responses and production of ROS in mitochondria. Taken together, these gene-profiling data support the concept that aging in the brain is associated with a state of heightened immune reactivity and oxidative stress accompanied by the accumulation of altered or misfolded proteins.

Several nuclear transcripts that declined in the brain during aging are required for mitochondrial function[23]. These included NADP transhydrogenase, ubiquinol–cytochrome-c reductase complex, subunit VIII of cytochrome-c oxidase and the γ and δ chains of ATP synthetase, all of which are either components of the mitochondrial electron-transport system or support its function. This profile indicates that mitochondrial function might be compromised in the aged brain, in agreement with the previous observation of altered respiratory rate and increased oxyradical production in mitochondria isolated from the brain of aged mice[26].

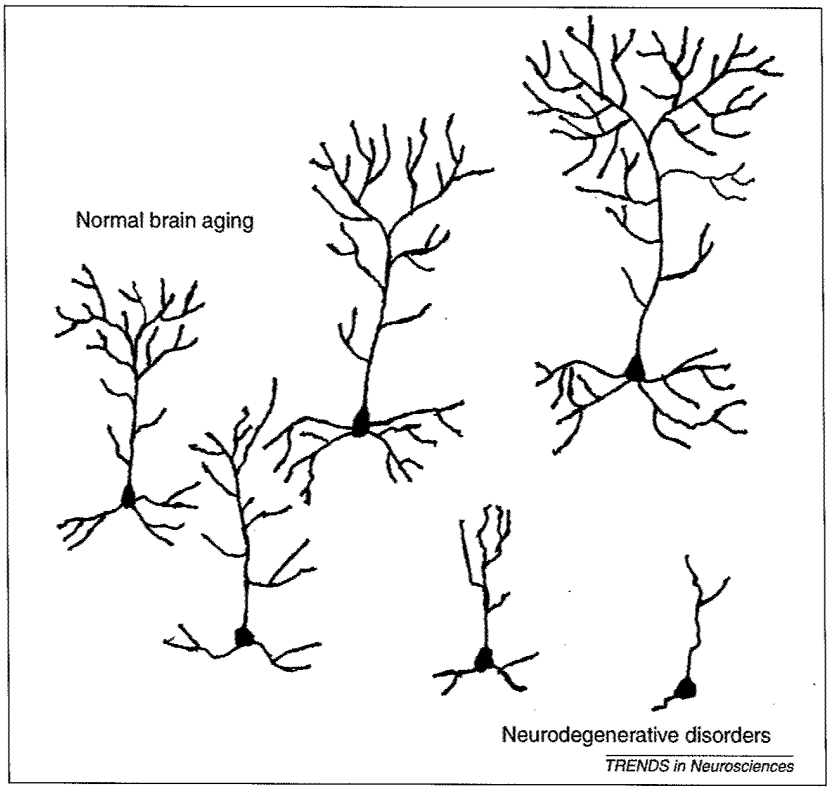

Figure 2. Morphological changes in cortical pyramidal neurons during aging

During normal brain aging, the dendritic arbors and synaptic contacts of individual neurons can expand to compensate for neuronal loss. In age-related neurodegenerative disorders dendritic arbors and synaptic connections are lost and compensation does not occur.

Age-related alterations in gene expression might represent adaptive mechanisms

It is not yet known which age-related changes in gene expression are detrimental and which are beneficial. On the one hand, based upon existing experimental data, it might be predicted that age-related decreases in levels of neurotrophic factors and increases in inflammatory mediators contribute to neuronal dysfunction and degeneration. On the other hand, the increase in levels of heat-shock proteins as the brain ages might help neurons cope with age-related increases in stress. Indeed, studies in *D. melanogaster* show that lines genetically selected for increased levels of hsp22 and hsp23 RNA have an increased lifespan and resistance to stress[27]. Similarly, *C. elegans* mutants with extended lifespans have increased stress responses to a variety of stresses including heat, ultraviolet radiation and pro-oxidants[28]. Therefore, although gene profiling provides a powerful initial screen to establish associations between levels of gene expression and a phenotype such as aging, cause–effect relationships can only be established through experimental manipulation of the individual genes.

Recent studies suggest that the nervous system might be an important 'master regulator' of lifespan. In *C. elegans* insulin-like signaling in neurons (via a receptor-tyrosine kinase called DAF-2 and a PI 3-kinase called AGE-1) can impose a limit on lifespan and, accordingly, mutations in these genes can increase lifespan[28,29]. In addition, mutations that cause defects in sensory perception can extend lifespan in *C. elegans*, possibly by altering the insulin signaling pathway[30]. Moreover, overexpressing the antioxidant enzyme Cu–Zn superoxide dismutase (Cu–Zn SOD) in motor neurons significantly increases the lifespan of *D. melanogaster*[31]. These invertebrate data should spawn new studies to determine the extent to which selective retardation of aging in the nervous system affects lifespan in mammals.

DR suppresses alterations in gene expression in the aging brain

DR prevents age-related alterations in gene expression

Because DR prevents or retards several features of CNS aging in rodents, including the age-associated increase in

the expression of GFAP and other markers of glial activation[20], the effect of DR on age-associated alterations in gene expression in mice has been determined[23]. Among the largest such age-related changes (>1.7-fold), 30% (34 out of 114) were either completely or partially prevented by DR. The effect of DR on age-associated alterations in gene expression was highly dependent on transcript class. For example, DR influenced only 20% (2 out of 10) of the observed decreases in expression of genes involved in neuronal growth and plasticity, whereas it prevented the induction of 50% (8 out of 16) and 65% (11 out of 17) of genes associated with the stress- and immune responses, respectively. The effects of DR on immune- and stress-related transcripts agree with studies indicating that both autoimmunity[2] and oxidative damage[32] is reduced in the brains of DR mice. Because these studies have only examined 5–10% of the mouse genome, it is possible that as many as 1000–2000 genes in the brain might undergo a >2-fold increase or decrease in expression during aging. Given that many of these genes are likely to belong to the same functional categories, a set of biomarkers representing elements from each class should provide a good tissue-specific panel to monitor aging.

DR induces a transcriptional reprogramming in the brain

In addition to reducing age-associated alterations in gene expression, DR shifted the expression levels of several transcripts that did not alter with age. A partial list is shown in Table 1 and a complete list of DR-mediated alterations in gene expression can be found at http://www.wisc.edu/genetics/CATG/prolla/data/aging/index.html. Compared with age-matched controls, DR induced the expression of 120 genes by 1.7-fold or higher in the neocortex, which represents 1.9% of the genes surveyed. One of the largest classes of transcripts induced by DR (9%) comprised growth and neurotrophic factors, including the developmentally-regulated homeobox genes, *Hox2.5*, *Hoxb3* and *Hoxa6*, all of which might be involved in neural development, and the gene encoding neuroserpin, a factor that promotes neuronal plasticity. Other transcripts in this general class have been linked to a neurotrophic function, such as *Tgfb3*, which encodes transforming growth factor (TGF) β3 and brain-derived neurotrophic factor (BDNF), which can protect neurons against excitotoxic and metabolic insults[33]. Perhaps one of the most interesting classes of transcripts induced in the brain of rodents under DR was that of genes involved in DNA synthesis or replication, such as thymidylate synthase, PCNA, MSH2, PUR-α and UMP synthase. The latter observation might be related to the increased neurogenesis observed in the brains of rodents under DR (Ref. 34). Taken together, gene expression analyses indicate that modulation of energy metabolism, oxidative stress, ion homeostasis and inflammatory signaling pathways by DR could profoundly affect brain aging in the mouse (Fig. 3).

DR and neurodegenerative disorders

Genetic and environmental factors in major neurodegenerative disorders

Life expectancy and the proportion of the population over the age of 65 are increasing rapidly, resulting in a dramatic increase in the incidence of age-related neurodegenerative disorders, particularly AD, Parkinson's disease (PD) and stroke. There has been considerable progress in the understanding of genetic and environmental risk factors for these disorders and in elucidating the molecular alterations that result in dysfunction and degeneration of neurons in the affected brain regions. Implicated in each of the chronic neurodegenerative disorders [AD, PD and familial amyotrophic lateral sclerosis (ALS)] are oxidative stress, metabolic abnormalities and protein aggregation[35,36]. In AD, degeneration and death of neurons occurs in brain regions such as the hippocampus and cerebral cortex that are involved in learning and memory processes. A hallmark of AD is the accumulation of amyloid plaques containing insoluble aggregates of a protein called β-amyloid (Aβ). Data from patients, cell culture and rodent models suggest that Aβ promotes neuronal degeneration by inducing oxidative stress and disrupting cellular calcium homeostasis[35]. Major advances in understanding the pathogenesis of AD have come from the identification of three genes encoding the amyloid precursor protein (APP), presenilin 1 and presenilin 2, mutations in which cause inherited, early-onset forms of the disease. When expressed in cells in culture or in transgenic mice, each mutation increases the production of neurotoxic forms of Aβ and increases the vulnerability of neurons to oxidative, excitotoxic and metabolic insults[35,37–39]. Environmental risk factors for AD include low educational attainment, traumatic head injury and, possibly, high-calorie intake[40,41]. Neurons might undergo a form of programmed cell death called apoptosis, in which a stereotyped molecular cascade involving death-inducing proteins, mitochondrial alterations and activation of proteases called caspases occur[42].

The motor dysfunction associated with PD results from degeneration of dopaminergic neurons in the substantia nigra. Although mutations in genes encoding α-synuclein and parkin have been linked to a small number of cases of PD, there appears to be a prominent environmental component to this disease, with exposure to toxins, head injury and high-calorie intake being probable risk

Table 1. Dietary-restriction-induced alterations in gene expression in the neocortex[a]

(fold)	SEM	Gene or protein	Function
↑ 2.0	0.3	IG α-chain C-region	Immunoglobulin chain
↑ 1.9	0.2	*RelB*	Inhibitor of inflammation
↑ 1.8	0.2	IFN-α_5	Modulator of inflammation
↑ 1.8	0.2	IFN-α_2	Modulator of inflammation
↑ 1.8	0.2	IFN-γ-induced Mg21	Modulator of inflammation
↑ 1.8	0.1	B-cell receptor	Antigen receptor
↑ 1.8	0.2	*Timp-1*	Metalloprotease inhibitor
↑ 1.8	0.1	*H-2K*(B) – α chain	Antigen presentation
↑ 1.8	0.2	*Lyt3.1*	MHC class I T-cell antigen
↑ 1.7	0.2	Macrophage CSF-1	Macrophage/microglial induction
↑ 3.1	0.3	NADPH oxidoreductase	Detoxification and biosynthesis
↑ 2.0	0.3	PERK	Attenuates protein translation
↑ 2.0	0.3	I-κBα chain	NF-κB inhibitor
↑ 1.7	0.3	*I-TRAF*	NF-κB inhibitor
↑ 1.9	0.3	Glucose-6-phosphate dehydrogenase	Pentose phosphate pathway
↑ 1.8	0.2	IF-2 homolog	Mitochondrial protein synthesis
↑ 1.7	0.3	Ferredoxin-NADP reductase	NADPH homeostasis
↑ 1.7	0.1	*Cox8h*	Mitochondrial ETS
↑ 3.0	0.3	*Bmp1*	Developmental factor
↑ 2.2	0.6	*Gas6*	Trophic factor
↑ 2.2	0.2	*Hox2.5*	Homeobox gene
↑ 1.9	0.2	*Hoxb3*	Homeobox gene
↑ 1.9	0.2	Neuroserpin	Neuronal plasticity
↑ 1.8	0.3	*Hoxa6*	Homeobox gene
↑ 1.8	0.1	*Bdnf*	Neurotrophic factor
↑ 1.7	0.2	*Ang*	Angiogenic factor
↑ 2.8	0.5	*Pur-α*	ssDNA binding protein
↑ 2.8	0.3	*Umps*	Pyrimidine synthesis
↑ 2.8	0.3	*Tyms*	Nucleotide synthesis
↑ 2.0	0.1	Pcna	DNA synthesis/repair
↑ 1.9	0.3	DP-1	Cell cycle regulator
↑ 1.8	0.2	Msh2	DNA mismatch repair
↑ 2.0	0.3	EF-1 α-2	Peptide elongation
↑ 1.7	0.2	Ribosomal protein S4 homolog	Ribosomal component
↓ 1.9	0.2	Multi(f)al. aminoacyl tRNA synthetase	tRNA synthesis
↓ 1.9	0.5	Valyl tRNA synthetase homolog	tRNA synthesis
↓ 1.8	0.2	EF-1-γ homolog	Elongation
↓ 1.8	0.2	60S ribosomal protein L10 homolog	Ribosome component
↓ 1.8	0.2	Threonyl tRNA synthetase	tRNA synthesis
↓ 1.8	0.2	Isoleucyl tRNA synthetase	tRNA synthesis
↓ 1.8	0.3	EF-2 homolog	Elongation
↓ 1.7	0.2	EF-1-Δ homolog	Elongation
↓ 1.7	0.3	EF-1-α-2 homolog	Elongation
↓ 2.2	0.5	*Mcp-5*	Inflammatory cytokine
↓ 1.8	0.3	*Stat6*	IL-4 signaling
↓ 1.8	0.2	IFN-receptor β-chain homolog	Modulator of inflammation
↓ 1.7	0.2	IFN-α	Modulator of inflammation
↓ 2.1	0.3	*Hsp27*	Heat shock factor/chaperone
↓ 2.1	0.3	NF-κB-p65	Oxidative stress response
↓ 1.8	0.4	Midkine precursor homolog	Response to neuronal injury
↓ 1.8	0.3	Cyclophilin A homolog	Chaperone
↓ 1.8	0.2	NF-κB-P100 subunit	Oxidative stress response
↓ 2.0	0.2	Phosphorylase-B-kinase γ subunit	Glycogen breakdown
↓ 1.8	0.2	Creatine transporter homolog	Creatine transport
↓ 1.8	0.3	Phosphoglycerate kinase 2	Glycolysis
↓ 1.7	0.2	Insulin receptor substrate 3	Insulin signal transduction
↓ 2.2	0.3	*Xpc*	DNA repair

[a]The data represent a comparison between 30-month-old dietary restricted (DR)-fed mice and control mice (n = 3).
Abbreviations: MHC, major histocompatibility complex; SEM, standard error of the mean.
For full gene names, see http://www.informatics.jax.org/searches/quick_gene_report.cgi
Key: Inflammatory response, Stress response, Energy metabolism, Growth/trophic factors, Protein synthesis, DNA synthesis/repair.

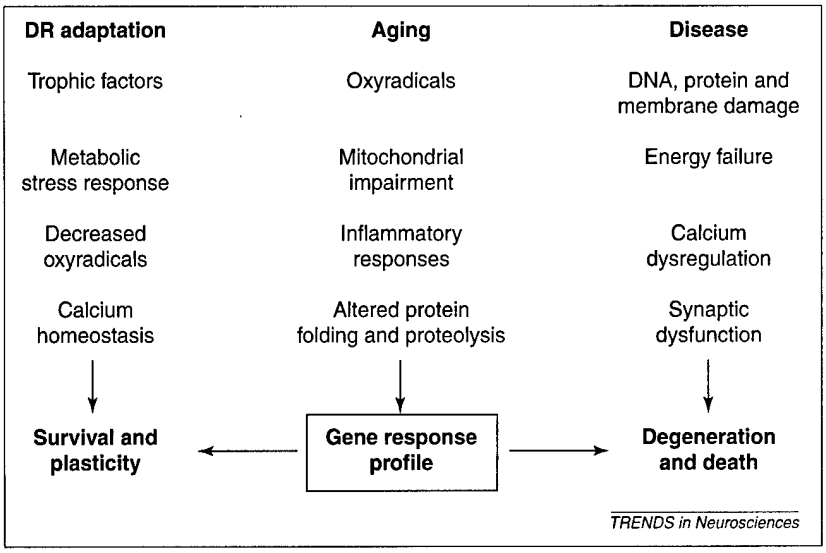

Figure 3. Roles of gene responses in determining resistance and vulnerability to age-related disease

Aging results in increased levels of oxidative stress, metabolic disturbances, inflammatory processes and dysregulation of protein catabolism. During successful aging, as promoted by dietary restriction (DR), cells adapt the expression of various genes resulting in maintenance and plasticity of neural circuits. Adaptive changes include those that suppress oxyradical production and stabilize cellular energy and calcium homeostasis. By contrast, unsuccessful aging, which might be facilitated by a high-calorie diet, results in neurodegenerative processes that manifest as disorders such as Alzheimer's and Parkinson's diseases.

factors[43]. Animal models indicate major roles for impaired energy metabolism, increased oxidative stress and apoptosis in the pathogenesis of PD.

The risk factors for stroke are similar to those for cardiovascular disease, with a genetic tendency towards hyperlipidemia, a diet that is high in calories and fat, and hypertension greatly increasing the risk. The symptoms of stroke patients are related to the extent of neuronal degeneration in the brain regions subjected to ischemia. Studies of animal models of stroke demonstrate the involvement of oxidative stress, overactivation of glutamate receptors and apoptotic biochemical cascades in the cell-death process[44].

DR is protective in animal models of neurodegeneration

Because advancing age is the major risk factor for AD, PD and stroke, a series of studies have been performed during the past three years to determine the effects of DR in animal models of these disorders. In the case of AD, three models have been studied. In one model, administration of the neurotoxin kainic acid (an excitatory amino acid) to rats results in selective degeneration of hippocampal pyramidal neurons and learning and memory deficits. Rats maintained on a DR feeding regime for 2–4 months are resistant to this effect (Fig. 4) and learning and memory in a water maze are preserved[45]. A second model consists of knockin mice in which a presenilin-1 mutation has been introduced, resulting in increased vulnerability of hippocampal neurons to excitotoxic and ischemic injury[39,46], and the third is APP-mutant mice which undergo age-dependent deposition of Aβ in the hippocampus and cortex and cognitive deficits[37,47]. In presenilin-1 mutant mice, hippocampal cornu ammonis (CA)1 and CA3 neurons of mice maintained on DR have increased resistance to excitotoxic injury compared with mice fed *ad libitum*[48]. The neuroprotective effect of DR was correlated with decreased levels of oxidative stress in the hippocampus. Thus, neurodegeneration promoted by a mutation that causes AD can be counteracted by DR. Surprisingly, when middle-aged APP mutant mice were subjected to DR on alternate days, they died within 2–3 weeks suffering from severe hypoglycemia[49]. Further analyses revealed abnormalities in responses of the APP mutant mice to a variety of stresses, including restraint stress and surgery characterized by abnormal neuroendocrine regulation of glucocorticoid and blood glucose levels. Thus, both presenilin-1 and APP mutant mice respond abnormally to stress, consistent with data obtained from studies of patients with AD (Ref. 50).

DR is also beneficial in experimental models of PD, Huntington's disease and stroke. The vulnerability of midbrain dopaminergic neurons to MPTP toxicity is decreased and motor function is improved in mice maintained on DR (Ref. 51). An animal model of HD involves administering the succinate dehydrogenase inhibitor 3-nitropropionic acid (3NP) to rats. Maintaining rats on a DR regime for several months before administration of 3NP increases the resistance of striatal neurons to 3NP and improves motor function[45]. DR was also beneficial in a rat model of stroke (Fig. 4) in which the middle cerebral artery is transiently occluded resulting in damage to the cerebral cortex and striatum supplied by that artery, and associated motor dysfunction[52]. Studies of human populations support a protective effect of DR against age-related neurodegenerative disorders. Studies of a large cohort of people living in New York City reveal that individuals with the lowest daily intake of calories have the lowest risk for AD (Ref. 41) and PD (Ref. 53). Moreover, the incidence of AD is decreased by more than 50% when genetically similar populations of Africans live in a community where they consume a reduced calorie diet[54].

In contrast to the beneficial effects of DR in models of AD, PD and HD, DR was ineffective in a transgenic model of ALS. ALS is a fatal disease characterized by progressive degeneration of spinal cord motor neurons resulting in progressive paralysis. A small proportion of ALS cases result from mutations in the gene encoding Cu–Zn SOD. However, transgenic mice expressing mutant Cu–Zn SOD did not benefit from DR and survived no longer than

transgenic mice fed *ad libitum*[55], suggesting that DR might not be effective in counteracting all genetically-based forms of neurodegenerative disorders. There are at least two explanations of why DR does not protect motor neurons in the mouse model. One possibility is that DR exerts different effects on gene expression in motor neurons and neurons in the brain. Another possibility is that DR does induce expression of neuroprotective proteins, such as growth factors and stress proteins, in motor neurons but that the pathogenic effects of the Cu–Zn SOD mutation involves a pathway that is not affected by DR. Further studies of DR in the transgenic ALS mice could help identify the specific molecular requirements for DR to counteract neurodegenerative disease.

Impact of aging and DR on the synapse

Synapses are highly specialized sites of interneuronal signal transduction that dictate the immediate functions and long-term plasticity of the brain. Structural and functional changes in synaptic connections that occur during aging might enable neuronal circuits to adapt to the increasingly adverse environment created by oxidative damage and metabolic compromise. Analyses of brains of individuals who aged successfully reveal that neuronal loss can be compensated for by an increased complexity of dendritic arbors of the remaining neurons[56] so that there is no net loss of synapses[57]. By contrast, synapses are highly prone to degeneration in age-related disorders such as AD, although this might be partially compensated for by increased size of the remaining synapses[58]. Receptors for each of the major signaling pathways in the brain are concentrated in synapses, including those activated by neurotransmitters, neurotrophic factors, cytokines and cell-adhesion molecules. Levels of several synaptic ligands and/or receptors can change during aging. For example, levels of dopamine D2 receptors, nerve growth factor receptors and BDNF decrease during aging in one or more brain regions[33,59]. Electrophysiological analyses of hippocampal slices from old and young rodents reveal changes in channels that regulate calcium influx and so might shift thresholds for the synaptic mechanisms that underlie learning and memory[60] (long-term potentiation and long-term depression). The specific molecular and cellular mechanisms that lead to alterations in synaptic function and structure have not been firmly established, but are likely to include oxidative stress and metabolic disturbances.

The possibility that abnormalities in signal-transduction pathways that normally regulate adaptive changes in synaptic structure and function contribute to dysfunction and degeneration of synapses in age-related disorders is indicated by data implicating activation of glutamate receptors in the pathogenesis of neurodegenerative disorders[61]. Populations of neurons in AD, PD, HD and stroke are also vulnerable to excitotoxicity, particularly under conditions of increased oxidative stress (e.g. exposure to Aβ in AD) and metabolic compromise (e.g. impairment of complex I in PD); glutamate receptor antagonists can protect neurons in animal models of each disorder. Activation of several synaptic signaling pathways, including those activated by neurotrophic factors, some cytokines, and integrins, can protect neurons against excitotoxic injury[33,62]. During successful aging, the latter synaptic signaling mechanisms are sufficient to guard against excitotoxic damage, whereas in neurodegenerative disorders the protective mechanisms are overwhelmed (Fig. 5).

Several lines of evidence suggest that DR can enhance synaptic function and increase the resistance of synapses to degeneration during aging in rodents. For example, age-related deficits in learning and memory are ameliorated in rodents maintained on DR (Ref. 63). In addition, DR prevents age-related deficits in long-term potentiation[64].

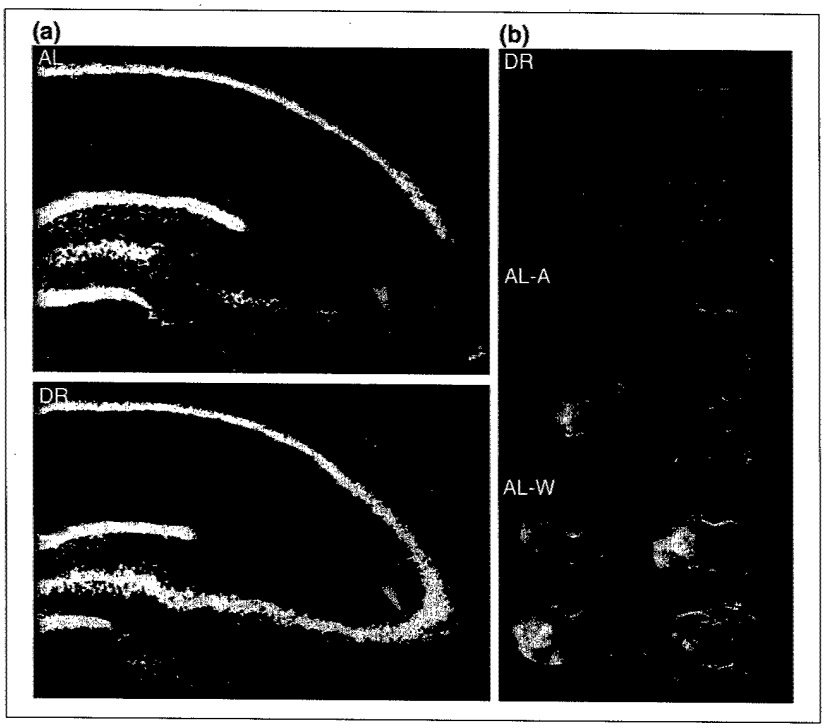

Figure 4. Dietary restriction increases the resistance of neurons in the brain to degeneration in animal models of Alzheimer's disease and stroke

(a) Cresyl-violet-stained coronal sections of hippocampus from rats given kainic acid, an excitotoxin that selectively damages hippocampal pyramidal neurons and results in impaired learning and memory ability. One rat had been maintained for three months on dietary restriction (DR) on alternate days (top), whereas the other rat had been fed *ad libitum* (AL, bottom). There is extensive loss of CA3 neurons in the rat fed AL, whereas very few neurons were damaged in the DR rat. **(b)** Sections of brain from three rats (four different coronal sections from each brain) subjected to transient occlusion of the middle cerebral artery as a model for stroke. The rats had been maintained on DR (upper) or fed AL; the brain in the middle panel is from an age-matched rat fed AL (AL-A) and that in the lower panel is from a weight-matched rat fed AL (AL-W). The brain sections were incubated with a dye that stains viable cells red and white areas in the sections correspond to regions where cells have died. There is considerable damage to striatal and cortical cells in the rats fed AL, but not in the rat subjected to DR.

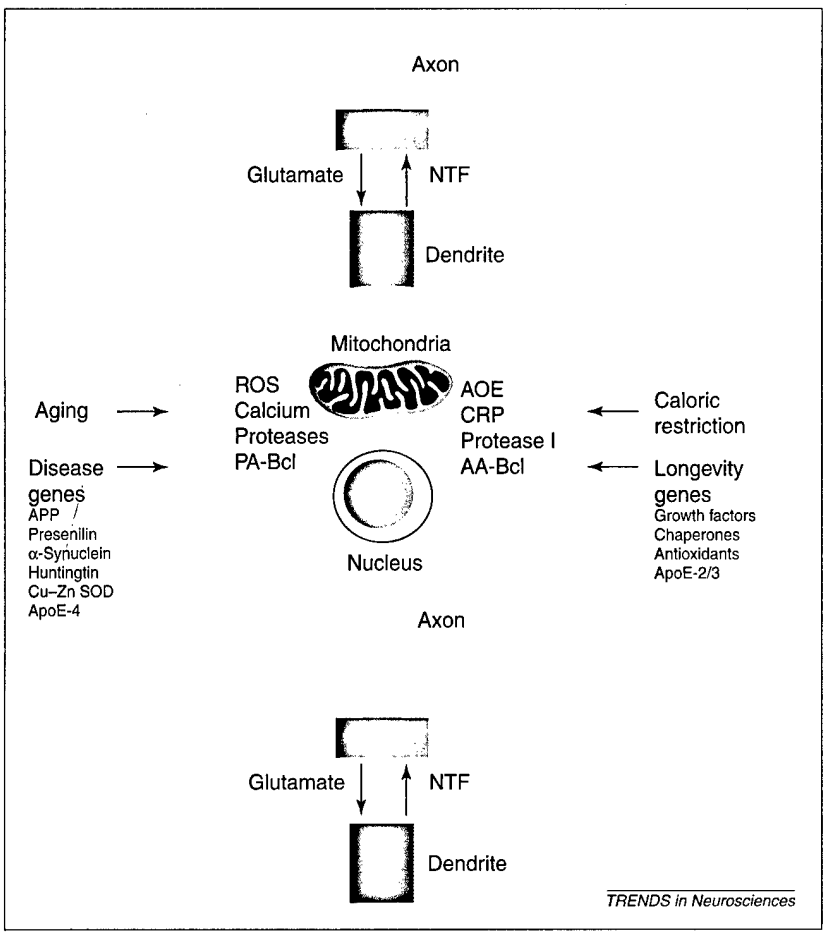

Figure 5. Signaling mechanisms that regulate neuronal plasticity and survival, and the effects of genotype, aging and dietary restriction

Changes that occur during aging (e.g. oxyradical damage and energy impairment) and specific genetic defects (e.g. mutations in the genes encoding the proteins indicated) promote oxidative stress, perturb cellular calcium homeostasis and activate apoptotic cascades in neurons. DR and 'good' genes, such as those encoding some growth factors, stress proteins and isoforms of apolipoprotein E, antagonize the effects of aging and 'bad' genes. Two prominent signaling pathways that might determine the outcome (normal brain aging or neurodegenerative disorders) during brain aging are those activated by glutamate and neurotrophic factors. Abbreviations: AA-Bcl, anti-apoptotic Bcl-2 family members; AOE, antioxidant enzymes; ApoE-4, apolipoprotein E4; APP, amyloid precursor protein; CRP, calcium-regulating proteins; DR, dietary restriction; PA-Bcl, pro-apoptotic Bcl-2 family members; NTF, neurotrophic factor; ROS, reactive oxygen species.

Moreover, in rats maintained on DR, levels of HSP-70 and glucose-regulated protein 78 (GRP-78) are increased locally in synaptic terminals, and synaptic terminals are more resistant to oxidative impairment of transport of glucose and glutamate across the cell membrane and exhibit enhanced mitochondrial function[65]. DR might, therefore, induce a preconditioning response that enables synapses to cope with the oxidative and metabolic stresses associated with aging.

Hormesis hypothesis for the beneficial effects of DR

DR induces a metabolic stress response

The ability of DR to increase the resistance of neurons in rodents to a broad array of insults correlates with changes in expression of several genes, among which those encoding chaperone proteins and neurotrophic factors stand out. Levels of HSP-70 and GRP-78 were increased in cortical, striatal and hippocampal neurons of DR rats compared with rats fed *ad libitum* (Fig. 6), whereas levels of HSP-60 were unchanged[51,52]. Whereas DR might reduce the expression of chaperones induced by oxidative stress owing to reduced steady state levels of oxidants as suggested by microarray data, it appears to induce chaperones and other protective factors involved in a 'metabolic stress response'. These might be protective against age-related dysfunction, as demonstrated by HSP-70 and GRP-78, which protect neurons against excitotoxic and oxidative insults[66,67]. This 'preconditioning' effect could contribute to the beneficial effects of DR in the AD, PD, HD and stroke models described previously. That a moderate energetic stress is sufficient to account for the neuroprotective effects of DR is further supported by studies showing that administration of 2-deoxy-D-glucose (a non-metabolizable analog of glucose) to animals fed *ad libitum* increases the resistance of neurons in PD and stroke models[51,52]. Interestingly, the beneficial stress response induced by DR and 2-deoxy-D-glucose in rodents can be detected at the level of synapses[68]. Long-term dietary supplementation with 2-deoxy-D-glucose in rats results in physiological changes resembling DR, including decreased plasma glucose and insulin levels[69] consistent with a shared cellular mechanism.

DR induces neurotrophic factors and neurogenesis

More recent studies show that levels of several neurotrophic factors, most notably BDNF, increase in hippocampal and cortical neurons of rats and mice maintained on a DR feeding regime[34,70] (Fig. 6). BDNF protects neurons against excitotoxic, oxidative and metabolic insults in various experimental models of neurodegenerative disorders[33]. BDNF and other neurotrophic factors have also been shown to exert beneficial effects on synaptic plasticity and might thereby facilitate learning and memory[71]. Increased BDNF production might be a necessary event in the neuroprotective effect of DR because infusion of a BDNF blocking antibody into the lateral ventricles of rats maintained on DR significantly attenuates the protective effect[70].

Work performed during the past decade has established that the adult mammalian brain contains populations of neural progenitor cells (NPCs) that are capable of dividing and then differentiating into neurons or glial cells in a process called neurogenesis. Such NPCs are most abundant in the subventricular zone and the dentate gyrus of the hippocampus[72] and might provide a reservoir that can be used to replace neurons or glia under

conditions of increased environmental demands or brain injury. Indeed, neurogenesis can be stimulated by ischemic and excitotoxic brain injuries[73,74], by physical exercise[75] or by rearing animals in an enriched environment[76]. The capacity of the brain for neurogenesis might decrease during aging[77] and DR has been shown to increase the number of newly generated neural cells in the dentate gyrus of the rat hippocampus[34]. Additional data in this study indicated a role of increased production of BDNF in the beneficial effect of DR on NPC. Collectively, the findings on the regulation of NPC by environmental factors, aging and DR are consistent with a hormesis mechanism in which DR stimulates brain cells to produce neurotrophic factors and other proteins that enhance the plasticity and survival of NPC and neurons.

Future directions

Gene expression profiling provides a powerful tool to identify global changes in gene expression that are associated with either successful aging or neurodegenerative disorders. However, these observations should be validated biologically to determine cellular localization and to verify a corresponding change in the levels of the encoded proteins. A further, crucial step is to move beyond identifying associations, and establish cause–effect relationships. Only this way can we determine which changes in gene expression are causally involved in age-related neuronal dysfunction and degeneration, and which changes are adaptive responses. The same applies to understanding the mechanisms whereby DR reduces the risk for age-related diseases. For example, are increases in levels of neurotrophic factors and chaperone protein sufficient to account for the beneficial effects of DR?

Although progress has been rapid in identifying genetic factors and biochemical cascades involved in the pathogenesis of age-related neurodegenerative disorders, molecular mechanisms of aging *per se* have been elusive. However, the involvement of oxidative stress and metabolic disturbances is repeatedly indicated. Oxyradical- and energy metabolism are highly complex and there are, therefore, many possible sites for dysregulation in aging and modulation by DR. Additional regulatory systems implicated in the aging process are those involved in cell proliferation and survival, and ion homoeostasis. Perhaps more than in any other type of organ, the function of the nervous system depends upon highly specific, intricate intercellular signaling networks whose regulatory mechanisms extend beyond gene transcription. It is, therefore, essential to understand such mechanisms at the level of protein interactions within individual cells, organelles and synapses. Proteomic technologies are improving[78] and, combined with various imaging methods, will reveal the molecular dynamics that maintain homeostasis and mediate plasticity in the brain. Understanding such molecular interactions and their genetic and environmental regulation will be a major challenge in the fields of brain aging and neurodegenerative disorders.

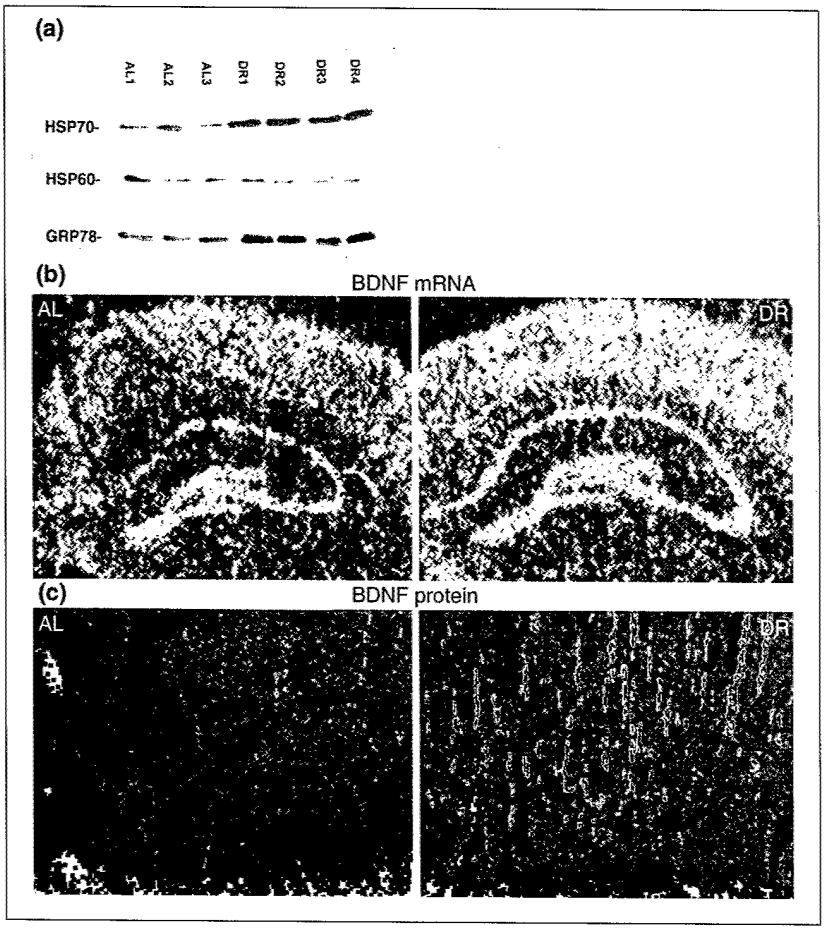

Figure 6. Dietary restriction increases levels of stress proteins and neurotrophic factors in the brain

(a) Immunoblot showing the relative levels of HSP-70, GRP-78 and HSP-60 in striatal tissue from rats maintained for three months on either AL or DR feeding regimes. **(b)** *In situ* hybridization autoradiograms of the relative levels of mRNA encoding BDNF in the hippocampus and overlying cerebral cortex in brain sections from rats maintained for three months on either AL or DR feeding regimes. DR increases mRNA encoding BDNF in CA1 pyramidal neurons and cerebral cortical neurons. **(c)** BDNF immunoreactivity in the cerebral cortex of rats maintained for three months on either AL or DR feeding regimes. BDNF concentrations increase in dendrites of many cortical neurons following DR. Abbreviations: AL, *ad libitum*; BDNF, brain-derived growth factor; CA, cornu ammonis; DR, dietary restriction; GRP-78, glucose-regulated protein 78; HSP-70, heat shock protein 70.

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Huntington's disease: new hope for therapeutics

Cynthia T. McMurray

Huntington's disease (HD) is one of eight progressive neurodegenerative disorders in which the underlying mutation is a CAG expansion encoding a polyglutamine tract. There are currently no cures or even effective therapies for HD. Effective strategies have remained elusive because little is known about either the mechanisms of expansion or the mechanism of polyglutamine-mediated neuronal death. However, recent advances in understanding the basic mechanisms of expansion and toxicity have renewed hope that a therapeutic strategy might someday be possible. Strategies effective in the treatment of HD are likely to be relevant in the treatment of a range of neurological and neurodegenerative disorders.

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Huntington's disease (HD) is one of eight dominantly inherited and progressive neurodegenerative disorders in which the underlying mutation is caused by a CAG expansion within the coding sequence of the affected gene (Fig. 1)[1,2]. Expansion causes disease when the CAG triplet in the mutated allele exceeds that of the normal range[1–3]. However, a feature that distinguishes trinucleotide expansion is the non-Mendelian form of inheritance[3]; the repeat number can grow in each successive transmission (Fig. 1a). Each CAG triplet codes for the amino acid glutamine. As the CAG repeat number grows, the growing polyglutamine tract produces an HD gene product (called huntingtin) with increasingly aberrant properties that cause the death of brain cells controlling movement, memory and behavior[1–3] (Fig. 1b). Above 36 CAG repeats, loss of brain cells (primarily in the striatum and cortex) causes the personality changes, cognitive decline and uncontrolled muscle movements (termed 'chorea') that are characteristic of the disease[1–3] (see also the Hereditary Disease Foundation's website at http://www.hdfoundation.org). However, onset and severity of symptoms are strongly influenced by the number of CAG repeats in the disease allele[1–3]. In individuals with 36 CAG triplets, symptoms can go unnoticed or develop mildly late in life. As the CAG triplet number grows, those affected develop more severe features at a younger age (Fig. 1a). Once symptoms begin, death usually occurs within 15–20 years[1–3].

There is currently no cure, or even an effective therapy, to offset the decline in mental and motor capacity suffered by those affected by HD. However, recent advances in understanding have provided new hope that a therapeutic strategy might one day be possible.

Therapy at the DNA level

The strong dependence of the character of the disease on the CAG repeat length has raised the possibility that stopping expansion at the DNA level might be an effective therapeutic strategy. Recent studies in mice have suggested that the inherited distribution of CAG repeat lengths of expanded transgenes remains stable after birth. However, at around 11 weeks of age, somatic expansion is observed in almost every tissue of the body and continues throughout the lifetime of the animal[4]. Age-dependent expansion is particularly prominent in the brain[4,5]. Thus, it is possible that tissues preferentially affected in disease contain huntingtin gene products with more and longer polyglutamine tracts. Although this phenomenon has not been confirmed in humans, there is intense interest in determining whether the age-dependent increases in the polyglutamine tracts length are related to the onset of disease.

Early efforts to stop expansion were hampered because the genetic mechanism was poorly understood. Recent findings, however, have confirmed that CAG

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expansion occurs during the repair of strand breaks and implicates the mismatch repair system in the mutation process[4].

Mechanism of expansion and possible therapeutic implications

It had been speculated that expansion occurs by polymerase slippage during cell proliferation or meiotic recombination (in sperm). By following CAG expansion in transgenic R6/1 mice, however, it has been observed that expansion occurs during post-mitotic phases in the brain and in the sperm of male animals. R6/1 mice harbor a single integrated copy of a transgene containing a CAG repeat in exon 1 of the human huntingtin gene (hHD)[5]. In germ cells, expansion occurs late in sperm development – at the point when cells called spermatids differentiate into mature sperm[4]. This is significant because spermatids are haploid cells that are both post-mitotic and post-meiotic. Therefore, CAG expansion cannot depend on mitotic replication or meiotic recombination and must arise from the repair of strand breaks. Break repair is also implicated as the mechanism in neurons – the age-dependent expansion is observed in the adult mouse brain at a time when neurons are also post-mitotic[4,6].

Expansion occurs when CAG/CTG loops are trapped in the DNA after gap repair synthesis (Fig. 2)[4]. It has long been recognized that expansion depends in some way on the ability of expansion-capable repeats to form secondary structures[3]. Then, at the site of the break, gaps can arise when CAG repeats form stable hairpin loops (Fig. 2). Repair of the gaps traps DNA loops, which are the precursors for expansion. DNA loops can also be trapped by slippage within the repeat region during repair-dependent, gap-filling synthesis[4] (Fig. 2).

A big surprise was the discovery that the mismatch repair system, the normal function of which is to remove mispaired bases and loops from DNA, plays a causative role in the mutation[4,7]. As shown by mating hHD (R6/1) animals with Msh2$^{-/-}$ animals, the absence of Msh2 completely abolished germ line expansion and age-dependent, somatic expansion[4,7]. It is suspected that an Msh2 complex binds and stabilizes the mispaired bases in the stems of the CAG hairpins[4]. Hairpins that form from CAG repeats contain an A–A mismatch base every third position between two Watson–Crick C–G pairs[3]. Stable hydrogen bonding within a contorted DNA template could abort a crucial step of recognition or coupling of the mismatch repair system[8].

The finding that loss of Msh2 attenuates expansion in animals provided the first evidence that expansion can be stopped *in vivo*, and has raised the hope that a therapeutic intervention of a repair complex could be used to attenuate, or at least delay, onset of disease. Although complete elimination of the Msh2 repair function is unlikely to be beneficial, the mechanism by which Msh2 is involved in expansion might reveal pathways and points of intervention that could be useful in a potential therapy. Additionally, it has been suggested that the mutational 'load' caused by age-dependent expansion could initiate a DNA damage response resulting in cell death. If this is correct, inhibition of damage-induced apoptosis could be therapeutic (see 'Caspase inhibition' later in the review).

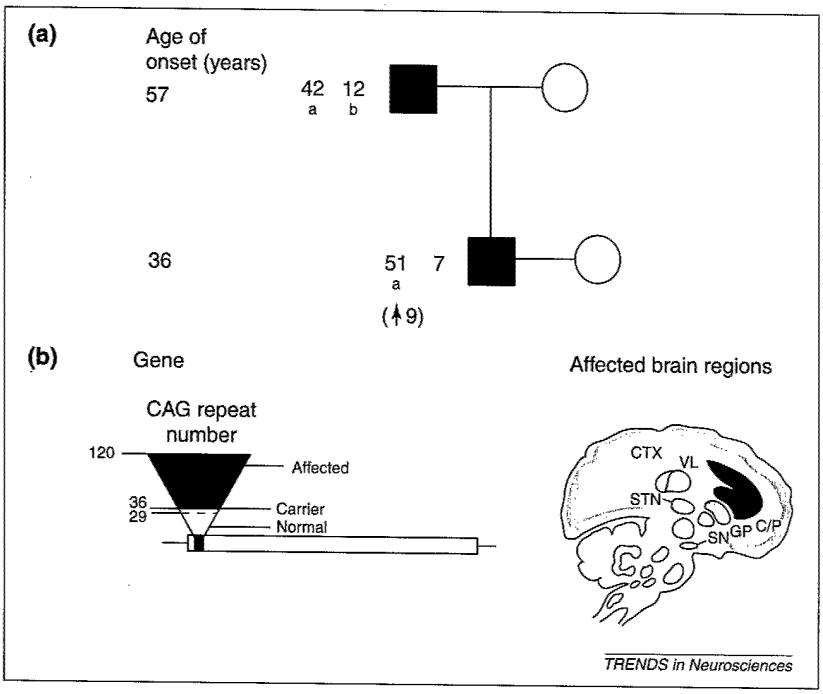

Figure 1. Effects of CAG expansion in the huntingtin gene

(a) Expansion in huntingtin decreases the age of onset in those affected. A representative two-generation pedigree of an HD family. Squares are males; circles are females. Red boxes indicate affected individuals. Open circles are unrelated spouses. Green numbers represent CAG repeat number in each allele of the affected family members. Small green letters indicate the alleles present in father-to-son transmission. The unlettered repeat number (blue) is a normal allele inherited from the mother. The number in brackets represents the size of the CAG expansion during inheritance. Age of onset of symptoms is indicated on the left in years. **(b)** The relationship between HD pathophysiology and CAG repeat number (left). In this schematic representation of the HD gene, the open bar represents the coding region of the Huntington's gene (called huntingtin); the lines indicate the non-coding portions of the gene; the small red bar indicates the position of the CAG repeat stretch located within the N-terminal portion of the coding sequence. The inverted triangle represents an increasing number of CAG repeats. The base of triangle, in white, represents unaffected individuals with 6–29 CAG repeats; dotted lines indicate unaffected carriers for disease with 29–35 CAG repeats and the red part of the triangle indicates affected individuals with 36–120 CAG repeats. Regions of neuronal loss in HD are shown on the right. Red regions indicate the major areas of neuronal loss in HD patients with 36–120 CAG repeats; these brain regions control movement. Abbreviations: C/P, caudate/putamen; CTX, cortex; GP, globus pallidus; STN subthalamic nucleus; VL ventrolateral thalamic nucleus; SN substantia nigra.

Therapy at the protein level: managing the toxic effects of the mutant huntington protein

Interfering with huntingtin-mediated aggregation

Stopping CAG expansion, even if successful, will at best prevent disease progression and diminish disease severity.

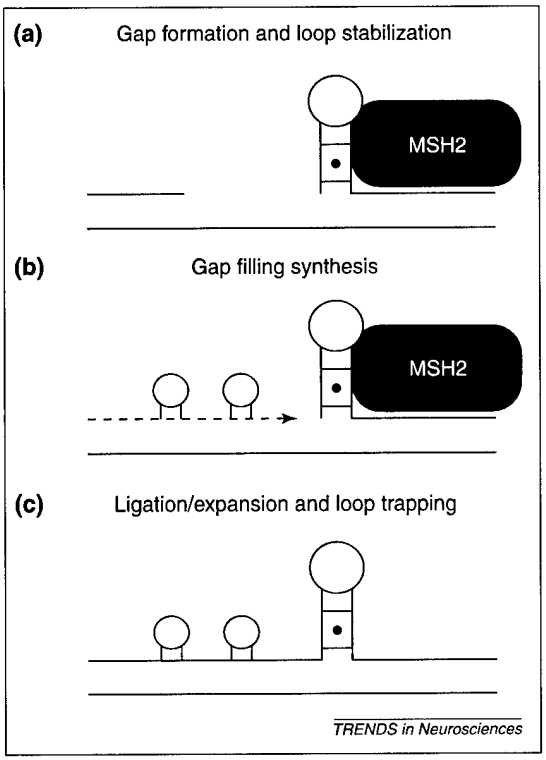

Figure 2. Model for expansion by gap repair

(a) After a strand break, gaps can form by folding of CAG/CTG repeats into hairpins. Hairpins comprising CAG repeats form a repeating unit of an A–A mismatch base every third position (dot in the hairpin stem) between two Watson–Crick C–G pairs (lines in hairpin stem). Msh2 might stabilize loop structures. **(b)** Consequently, Msh2 binding prevents the reannealing of the complementary strands at the break site and increases the lifetime of the gap, forcing its repair. As haploid cells contain only one complement of the chromosomes, repair is not possible through genetic exchange with a homologous chromosome or a sister chromatid. Repair can occur through a simple 'fill-in' reaction or by gene conversion in which the DNA loop invades a CAG repeat region in a non-homologous chromosome. Loops of DNA can be introduced into the DNA either at the site of the break by folding of CAG/CTG repeats into secondary structure or at the break or during gap repair synthesis (b). **(c)** Gap filling synthesis followed by ligation results in loop trapping and gain of DNA at the site of the break (bottom).

Therefore, more emphasis has been placed on developing therapeutic strategies to offset or prevent toxicity induced by the mutant huntingtin protein. Mutation analysis and transgenic animal models for disease have unequivocally identified the expanded polyglutamine tract as key in toxicity[1–3]. As with the expansion, the mechanisms by which the polyglutamine tracts kill neurons are unclear and the function of huntingtin protein is unknown. Polyglutamine regions self-associate to form polar zippers (β-strands that assemble into sheets or barrels by hydrogen bonding)[9], promoting aggregation. In HD-affected areas of the caudate and cortex, immunochemical detection reveals that mutant huntingtin protein forms high molecular weight complexes and inclusion bodies[1–3,5]. Despite an incomplete understanding of toxicity, it is generally accepted that aggregation and sequestration of cellular targets are causative factors[1–3,9]. Consequently, antibodies and a number of small molecule inhibitors are being developed to block aggregation. For example, when co-expressed with a truncated form of the mutant huntingtin protein, single-chain sFv antibodies have been shown to reduce the number of visible aggregates in mammalian cells[10]. Similarly, small molecules and peptides inhibitors[11–13] of huntingtin-mediated aggregation are being developed that specifically bind to huntingtin, reduce aggregation and improve cell survival. To date, the testing of new molecules has been limited to *in vitro* culture systems. However, future *in vivo* studies will determine whether modulation of aggregates represents a viable approach to treatment for HD.

Identification of specific targets of huntingtin-mediated aggregation has also suggested new avenues for therapy. Many cytoplasmic[14] and nuclear proteins[15] are reported to associate with normal and mutant huntingtin. Cytoplasmic targets include vesicular trafficking motors, ubiquitin-conjugating enzymes, actin-organizing proteins, and microtubules. Nuclear targets include p53 and CREB binding protein. Although loss of any of these targets might be deleterious to cell function, one in particular has generated intense interest. The mutant huntingtin protein can sequester its normal counterpart in the cell. Huntingtin protein has essential functions during development[16] and throughout the life of an animal[17]. For example, it has recently been reported that huntingtin can regulate transcription of brain-derived neurotrophic growth factor (BDNF)[18]. BDNF is particularly important for the growth of striatal neurons; consequently, loss of BDNF, owing to huntingtin-mediated aggregation, could contribute to neuronal death. These data suggest that treatment with BDNF could improve neuronal survival. In addition, sequestration of normal huntingtin could abolish a recently reported anti-apoptotic function of the normal protein that blocks activation of procaspase-9 (Ref. 19). Therefore, loss of the normal huntingtin protein by the mutated gene product could result in a 'functional knockout'. Indeed, conditional knockout in mice of the normal huntingtin protein after birth in mice results in a progressive neurodegenerative phenotype similar to HD (Ref. 17). If correct, future efforts could focus on how to replace the function of the normal huntingtin in a manner that is not defeated by aggregation of the mutant protein.

Cellular processes suspected of being altered by polyglutamine-dependent aggregation have resulted in the testing of several therapeutic strategies for HD. All of these have met with modest success.

Replenishing energy metabolism owing to oxidative stress

Disruption of mitochondrial function and glucose metabolism has been proposed to mediate neuronal death in many neuropathological diseases, including HD (Ref. 20). In human HD patients, magnetic resonance imaging confirms that creatine (which is a free-radical scavenger, a substrate for the enzyme creatine kinase and a precursor for ATP) is depleted[21]. Similarly, in cells expressing the mutant huntingtin protein, mitochondria do not readily take up cationic dyes that depend on intact charge gradients[22]. These data indicate that mitochondrial membrane potential is impaired because of expression of the polyglutamine protein. In rats, systemic administration of the mitochondrial complex II inhibitor, 3-nitropropionic acid, causes neurobehavioral and pathological abnormalities consistent with HD (Ref. 23) and, in HD patients, the caudate has severe deficiencies in mitochondrial complexes II and III (Ref. 24). Finally, in affected striatal and cerebral regions of the brain, glucose metabolism is decreased and precedes bulk tissue loss in HD patients[24]. Taken together, these data point to impairment of mitochondrial function as contributing factor in HD.

If mitochondrial deficits and ATP depletion play a role in HD, then replenishing impaired energy metabolism might offset toxicity by restoring ATP levels. Indeed, the survival rate of animals treated with dietary creatine does increase in both R6/2 transgenic mice[25] (which are the same as R6/1 mice but with a larger CAG tract[5]) and in mice subjected to systemic administration of 3-nitropropionic acid[26]. In both sets of animals, creatine not only improved survival but also delayed striatal atrophy and the formation of neuronal inclusions[25,26]. Despite improvements in the animals, supplementation with creatine has not yet proven effective in offsetting disease progression in human clinical trials[27]. Efficacy was also absent in an earlier study of another free-radical scavenger, OPC-14117 (Ref. 28). In both cases, however, the compounds were well tolerated with no adverse side-effects. Studies with larger patient groups are planned for creatine and clinical trials are now underway to evaluate the efficacy of co-enzyme Q, another free-radical scavenger that improves energy production in mitochondria[26]. However, even if these agents are protective they are unlikely to be a permanent cure or therapy, but might prove efficacious in combination with other treatments.

Glutamate excitotoxicity

Among the affected cells in HD are specialized brain cells in the striatum called medium spiny neurons[29]. These neurons contain many small 'spines' that are rich in excitatory N-methyl-D-aspartate (NMDA) receptors. As expression of mutant huntingtin has recently been reported to enhance excitotoxic death in cultured cells[30], trials are now underway to study the effects of glutamate receptor blockers in HD patients. Clinical trials for lamotrigine have already been completed[31]. Lamotrigine is an anti-epileptic drug that blocks voltage-gated sodium channels, thus inhibiting glutamate release[31,32]. Although lamotrigine was successful in reducing chorea in patients, it failed to affect disease progression[31,32]. Currently, trials are underway to test the efficacy of remacemide[32], another blocker of this receptor-mediated, excitatory pathway.

Caspase inhibition

Damaged cells can be removed by initiating a programmed pathway to cell death mediated by caspase activation[33]. It has recently been suggested that huntingtin-mediated aggregation might induce activation of important initiator caspases 9 (Ref. 19), -8 and -10 (Ref. 34). Activated caspases 8 and 10 appear to be recruited to the insoluble fraction in homogenates derived from HD brains[34]. Initiator caspases are responsible for cleavage and activation of downstream effector caspases, such as caspase 3 (Ref. 33). Interestingly, caspase 3 cleavage of the mutant huntingtin protein generates a small, N-terminal peptide containing the expanded polyglutamine region, and cells transfected with this N-terminal fragment form nuclear inclusions and undergo apoptosis[35]. Similarly, mice expressing a truncated N-terminal fragment of huntingtin display inclusions and a progressive neurological phenotype, although neuronal loss is not necessarily observed[5,36]. These data have given rise to the 'toxic peptide' theory of pathogenesis in which a small N-terminal fragment containing the expanded polyglutamine region must be generated to mediate toxicity. Unambiguous proof that caspase cleavage of huntingtin occurs *in vivo* has not yet been demonstrated and the full-length mutant protein is not easily degraded in transgenic animals[29]. Thus, it is still not known if it is the full length or truncated form of huntingtin that initiates early events of HD pathophysiology.

If caspase activation occurs early enough in disease progression then disease onset could be blocked by the use of caspase inhibitors, which might provide protection by blocking a general cell death pathway, by preventing the formation of toxic N-terminal fragment, or both. Indeed, several attempts to rescue the HD phenotype in mice by using caspase inhibitors have been reported. For example, Ona and colleagues reported that caspase 1 is activated in the brains of both mice and

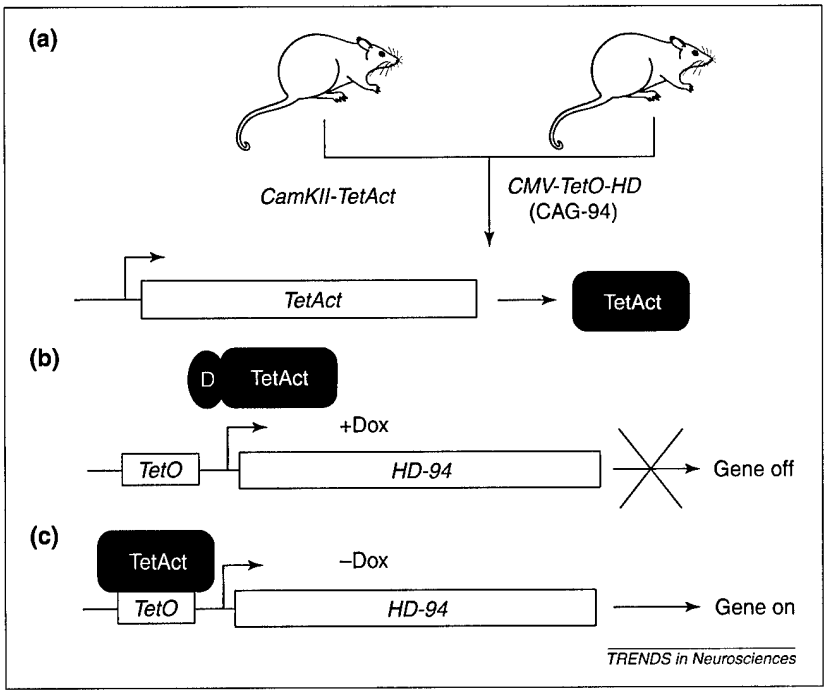

Figure 3. 'Tet-off' regulation of huntingtin in the mouse

(a) Two mouse lines were generated and bred. The first contained the tetracycline transactivator (TetAct) under control of the calmodulain kinase II promoter (CamKII). TetAct is a fusion protein between the tetracycline-responsive repressor and the activation domain of the herpes simplex VP-16 transcriptional activator. TetAct binds to the tetracycline-responsive operator sequence and activates a linked gene. The second mouse contained a truncated, recombinant HD transgene (CMV-TetO-HD). The transgene contained only exon 1 of the human huntingtin gene, with a CAG repeat of 94 driven by a cytomegaloviral promoter (CMV) engineered to contain a tetracycline-responsive operator sequence (TetO). Progeny of the breeding contain both transgenes and are capable of antibiotic regulation of the HD transgene expression. **(b)** To regulate HD expression, progeny mice are exposed to doxycycline (Dox) in the drinking water (+Dox). Dox is a stable derivative of tetracycline that binds to the TetAct and causes dissociation from the TetO. As TetAct is required for CMV-TetO-HD expression, dissociation abolished production of the toxic huntingtin gene product (Gene off). **(c)** When Dox is removed from the drinking water (–Dox), TetAct binds to the operator and enables expression of the toxic huntingtin gene product (Gene on). The black circle is doxycycline (D); open bars represent the cDNAs of the respective genes; lines represent the promoter elements; arrows represent the transcriptional strat sites.

humans with HD (Refs 37,38). R6/2 transgenic mice treated by intracerebroventricular injection of caspase 1 inhibitors[37] or 3-propionic-acid-challenged animals that express a dominant-negative caspase-1 (Ref. 38), displayed improved motor function and significantly delayed onset of symptoms and mortality. The N-terminal human huntingtin fragment present in R6/2 mice is an approximately 75-amino-acid protein, in addition to the glutamines, and contains no caspase 1 cleavage sites. Therefore, caspase 1 inhibition cannot be acting by preventing N-terminal cleavage of the mutant huntingtin protein.

It has therefore been speculated that blocking a general programmed death pathway might improve survival of affected brain cells. Indeed, clinical trials are planned to test the efficacy of minocycline, an inhibitor of cell death pathways that has improved survival in animals[39]. Minocycline is a derivative of the antibiotic tetracycline that crosses the blood–brain barrier and inhibits caspases 1 and 3 (Ref. 39).

Like creatine treatment, caspase inhibition appears promising as a therapeutic approach, it merely delays disease progression in animals, being unable to prevent it. Thus, these compounds are not expected to cure HD; rather, it is hoped that they will improve symptoms and survival.

The approaches discussed above have in common a strategy to offset or to manage the toxic effects produced by the expanded huntingtin protein. Recently, however, several approaches have been aimed at cure or prevention of disease.

Transplantation

Polyglutamine aggregation leads to the death of several neurons. To offset the severe phenotype that ensues, surgical strategies have been developed in which transplantation of embryonic stem cells replaces lost neurons in the striatum[40]. Embryonic grafts placed in quinolinic-acid-treated animals improved motor functions such as paw reaching. Furthermore, neural precursors develop synaptic connections and express neural antigens and many markers of mature differentiation[41]. These studies suggest an exciting avenue for therapeutic intervention in severe cases. The success of grafting is sensitive to the age of the donor, the time of the graft placement and the degree of neuronal loss in the host[40,41], and can be improved by treating animals with caspase inhibitors[42]. This approach is highly invasive and will be useful only at later stages of disease progression. However, if effective, defective neurons would be replaced with normal ones that lack the mutant gene.

Inhibition of protein expression from the mutant allele

An ideal approach would be to eliminate the expression of the mutant protein before toxic effects occur. Such an approach could prove effective because function does not appear to be sensitive (within limits) to the amount of expressed gene product in HD patients. Individuals can vary by as much as 50% in their huntingtin protein content without developing disease (Ref. 36 and references therein). Because normal huntingtin is required for development[16,17], however, inhibition of the mutant allele is expected to be beneficial only if expression from the normal allele is preserved. Few anti-gene or antisense strategies have been reported[43,44]. Attempts using oligonucleotides specifically to reduce the expression levels of the mutant allele have been limited to cell culture and have met with limited success. For example, antisense oligonucleotides targeting the methionine initiation codon and exon 1 (the –25 to +35 region of the

promoter) can inhibit expression of a stable incorporated green-fluorescence-huntingtin in PC-12 cells to roughly half that of untreated cells[43]. Although these studies report successful reduction in the protein levels, whether a reduction of 50% is sufficient to rescue cell survival during long-term culture remains to be seen. A larger issue (that also remains to be tested) is whether this approach will be effective in targeting neural tissue in whole animals or humans.

Although testing of the antisense and anti-gene approaches is in its infancy, recent studies confirm that selective inhibition of the expanded Huntington's allele is likely to be an effective strategy[36]. Using a 'tet-off' regulatable huntingtin transgene (Fig. 3), Yamamoto and colleagues[36] were able to control the expression of the mutant truncated protein in mice. In the absence of doxycycline in the drinking water ('gene on' condition), these animals developed inclusion bodies, progressive clasping response, tremors, general brain atrophy, an increased ventricular size and reactive astrocytosis[36]. All of these effects evolved between 3 and 18 weeks of age. However, restoration of doxycycline (2 mg ml^{-1}; 'gene off' condition) at 18 weeks of age had, by 34 weeks, reversed the clasping phenotype, reduced the incidence of inclusions to nearly that of control mice, and attenuated or improved neuropathology. These data in mice provide important proof of principle that specific inhibition of the expanded huntingtin allele can indeed reverse the disease phenotype if the normal gene expression is maintained. However, the identification of a small molecule that can effect selective inhibition *in vivo* still remains to be achieved. This might be no easy task because the mutant and normal huntingtin alleles have few sequence variations to target outside of the CAG tract length.

Carriers of the expanded HD allele can be genetically identified long before clinical symptoms develop. Early detection and late onset of disease render HD patients particularly well suited for effective therapeutic intervention if this is developed. Although effective therapy is not yet possible, rapid advances in the understanding of the basic mechanisms of disease are leading to expanded approaches towards therapeutic strategies, and to renewed hope for a cure.

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Humoral autoimmunity as a mediator of CNS repair

Allan J. Bieber, Arthur Warrington, Larry R. Pease and Moses Rodriguez

Autoimmune responses directed against the central nervous system (CNS) have generally been considered pathogenic in nature. Although there are several well understood conditions in which this is the case, there is also a growing body of experimental evidence to show that both the cellular and humoral immune responses can promote tissue repair following CNS injury and disease. Our laboratory has used a mouse model of chronic demyelinating disease to characterize a class of polyreactive IgM autoantibodies that react with oligodendrocyte surface antigens and promote myelin repair. By screening a large number of human monoclonal antibodies, we have found that IgM antibodies that react with CNS tissue are relatively common. Autoreactive IgM antibodies might constitute an endogenous system for tissue repair, and therefore these antibodies could be of value as therapeutic reagents.

The central nervous system (CNS) is often considered a site of 'immune privilege', based, in part, on the physical separation of CNS tissue from peripheral immune function by the blood–brain barrier. However, the isolation of the CNS is often imperfect, and there are many examples in human disease and in animal models of disease in which both the cellular and humoral branches of the immune system interact with the CNS.

Evidence for CNS tissue repair mediated by the cellular branch of the immune system has recently been reviewed[1,2]. Here, we review recent work demonstrating that elements of humoral immunity might also play a role in tissue repair.

Autoantibodies as pathogenic agents

The existence of pathogenic autoantibodies is well-established for several peripheral neurologic syndromes, including myasthenia gravis, Lambert–Eaton syndrome, Guillain–Barré syndrome and acquired neuromyotonia. Questions concerning antibody-mediated central nervous tissue injury are always complicated by issues of blood–brain barrier permeability, but antibody involvement is suspected in both Ramussen's and Bickerstaff's encephalitis[3].

The involvement of pathogenic autoantibodies in a particular disease has generally been defined based on several lines of experimental and clinical evidence. Antibodies to a defined target should be present in the majority of patients with the disease. The presence of these antibodies is often demonstrated by using purified antibodies as immunostaining reagents on tissues that express the target antigen. Immunization with the target antigen should induce the disease in experimental animals, and passive transfer of antibody to non-immunized animals, or transfer of antibody from patients with the disease, should also induce disease. Reduction of serum antibody levels, either by plasma exchange or by immunosuppression, usually leads to clinical improvement, and rising antibody levels following these treatments are mirrored by a return of clinical symptoms.

Criteria such as these can define a role for pathogenic antibodies in the development of a disease. We have applied many of the same criteria to define autoantibodies that

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promote tissue repair in an animal model of multiple sclerosis (MS), the most common human CNS demyelinating disease. Before we describe these observations, we will first consider data on the role of autoantibodies in human MS.

Autoantibodies in MS

Elevated concentrations of immunoglobulin (oligoclonal bands) in the cerebral spinal fluid have long been used as a diagnostic marker for MS, and were originally interpreted as evidence for the involvement of the humoral immune response in the pathogenesis of MS. There have been many attempts to identify pathogenic antibodies in the serum of MS patients, and antibodies that are specific for a variety of myelin proteins and antigens have been detected, including antibodies against many of the major myelin proteins, such as myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG) and myelin-associated glycoprotein (MAG). However, there is little direct evidence that these antibodies are actually involved in the pathogenesis of MS, and the presence of these antibodies is not highly specific for the disease; antibodies to many of these antigens are also found in the serum of healthy individuals or in patients with neurological diseases other than MS.

Although there is little direct evidence to support a role for humoral immunity in the pathogenesis of MS, the possibility is supported by observations in animals with experimental autoimmune encephalitis (EAE). Immunization with myelin components such as MBP or PLP, produces EAE, a T-cell-mediated inflammatory disease in which demyelination is often minimal. However, if antibodies to MOG are injected after induction of EAE, the severity of the disease is dramatically increased and large demyelinating lesions develop[4–6]. MOG autoantibodies have been detected in association with disintegrating myelin in both human MS patients and in a marmoset model of EAE (Ref. 7). These observations support the notion that autoreactive antibodies have the potential to induce or exacerbate demyelinating disease.

Antibodies against myelin can promote CNS remyelination

A direct demonstration that autoreactive antibodies can enhance endogenous myelin repair came from studies in this laboratory using Theiler's murine encephalomyelitis virus (TMEV) to induce chronic demyelinating disease in mice[8]. After intracerebral infection with TMEV, the virus replicates in the gray matter of the brain, resulting in acute encephalitis that is resolved in 14–21 days, and which is followed by chronic viral persistence in spinal cord white matter. Persistent TMEV infection eventually leads to chronic demyelination and progressive loss of motor function, a clinical pattern that is very similar to that observed for progressive MS in humans. Myelin pathology in TMEV-infected mice is immune-mediated, with chronically infected animals demonstrating a wide range of disease phenotypes, depending on their specific genetic background. In the SJL strain, demyelination is evident within 30 days after infection, and by 1–3 months the animals begin to develop neurological deficits, such as spasticity and gait abnormalities, weakness of the lower extremities and bladder incontinence. Paralysis eventually occurs by 6–9 months. Spontaneous repair of damaged myelin is common in many mouse strains, but is relatively limited in the SJL strain; often less than 10% of the total demyelinated lesion area is repaired. The relative absence of spontaneous repair makes this an excellent model for the study of strategies to promote endogenous remyelination.

Our initial observation of a beneficial humoral immune response occurred when chronic TMEV-infected mice were immunized with spinal cord homogenate (SCH) in incomplete Freund's adjuvant. Histological examination of spinal cord lesions from immunized animals revealed substantial CNS remyelination compared with control animals that had been treated with adjuvant alone. Passive transfer of antiserum[9] or purified immunoglobulin[10] from uninfected animals that had been immunized with SCH also enhanced remyelination, demonstrating a beneficial role for the humoral immune response against SCH in promoting myelin repair.

To further explore the nature of this beneficial immune response, hybridomas were generated from SJL mice following SCH immunization, in an attempt to identify monoclonal antibodies (mAbs) that promote remyelination. Two mouse mAbs that enhance remyelination were subsequently identified and designated SCH94.03 and SCH79.08, respectively. Both antibodies are polyreactive IgM antibodies and both bind to antigens expressed on the surface of oligodendrocytes, suggesting that the remyelination-promoting activity of these antibodies might involve direct stimulation of myelin-producing cells[11]. Subsequently, four additional oligodendrocyte-specific mouse IgM antibodies were characterized and shown to promote CNS remyelination[12].

We have used oligodendrocyte binding as a screening assay for the identification of candidate human mAbs that might promote remyelination and therefore have potential as therapeutic reagents[13]. As a source of human mAbs, we used serum-derived human monoclonal IgMs (sHIgM) and serum-derived human monoclonal IgGs (sHIgG) isolated from patients with monoclonal gammopathy, a relatively common condition characterized by high concentrations of monoclonal serum antibody. We tested

52 sHIgMs and identified six that bound to the surface of morphologically mature rat oligodendrocytes in culture, whereas none of the 50 sHIgGs bound. The oligodendrocyte-binding sHIgMs were tested *in vivo* and two of these (sHIgM22 and sHIgM46) were found to have remyelination-promoting activity similar to that observed with the mouse monoclonals.

Figure 1a shows a well-remyelinated lesion from an animal treated with a 1 mg intraperitoneal injection of a serum-derived human monoclonal IgM, and an unremyelinated lesion from the saline treatment group (Fig. 1b). Histological examination of spinal-cord sections five weeks after treatment with either sHIgM22 or sHIgM46 antibodies revealed significantly greater repair of the lesioned area (17.06% and 27.12%, respectively), compared with animals treated with saline (6.74%) (Ref. 13). In general, increases in remyelination of approximately 3–5-fold are observed after treatment with remyelination-promoting antibodies. We estimate that this represents at least 30 000–50 000 remyelinated axons in an antibody-treated animal.

We have tested approximately 40 different mAbs, of varied isotype and with varying binding specificities, for their remyelination-promoting potential in TMEV-infected mice, and we have never observed a significant increase in the total demyelinated-lesion area following antibody treatment. This observation demonstrates that antibodies with pathogenic potential are relatively uncommon for this model system. We have tested antibodies against MOG and galactocerebroside (GalC) that have previously been reported as pathogenic in other experimental systems[4,6,14,15], but have not observed significant increases in the extent of demyelinated lesions following five weeks of antibody treatment. One antibody against GalC, mAb O1, actually promotes repair in this system[12]. The reasons for these differences are unclear, but there could be some species- and model specificity to the observed effects of these antibodies. It is worth noting, however, that the patients from whom sHIgM22 and sHIgM46 were isolated have very high levels of oligodendrocyte-reactive antibodies in their serum, but have shown no signs of neurological disease.

None of the antibodies that induce remyelination react with Theiler's virus, and treatment with these antibodies does not decrease virus levels in infected animals. Therefore, virus neutralization does not explain the tissue repair observed following antibody treatment.

Characteristic properties of remyelination-promoting antibodies

As outlined above, there are several properties that have been used to define certain autoantibodies as pathogenic agents. Similar criteria can be applied for the definition of autoreactive antibodies that are involved in tissue repair.

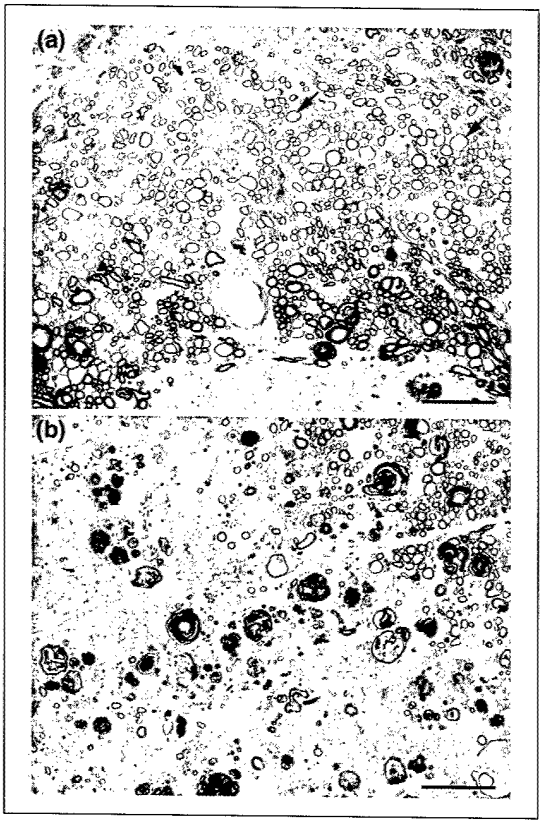

Figure 1. Remyelination of Theiler's murine encephalomyelitis virus (TMEV)-induced lesions

SJL mice with TMEV-induced demyelinating lesions were treated with either **(a)** the human monoclonal antibody sHIgM22 or **(b)** saline. Spinal cord cross-sections were stained for myelin with p-phenylenediamine. Remyelination of lesions is significant after five weeks of treatment with a remyelination-promoting antibody (a). Remyelinated axons (arrows) are identified by their relatively thin myelin sheaths compared with normal myelin. Thicker and darker-staining normal myelin sheaths are visible at the bottom of panel (a) and in the upper-right corner of panel (b). Animals treated with saline have many demyelinated lesions in the spinal cord; disintegrating myelin sheaths and myelin debris are clearly apparent (b). Scale bars, 25 μm.

Recognition of appropriate tissues or molecules is an important defining characteristic. All of the antibodies that promote remyelination bind to antigens on the surfaces of oligodendrocytes, suggesting that these antibodies might function through direct stimulation of the myelin-producing cells. However, these antibodies are highly polyreactive and recognize a variety of chemical haptens and proteins when binding is assayed by enzyme-linked immunosorbent assay. They also recognize intracellular proteins when used to stain a variety of permeabilized cells, but bind to a much more limited array of antigens on the surfaces of living oligodendrocytes. The oligodendrocyte surface antigens that are bound by several of these antibodies have been characterized, and are generally lipid or carbohydrate in nature rather than cell surface proteins[12,16]. Surface staining of a cultured oligodendrocyte with a human serum IgM is shown in Fig. 2(a).

Remyelination-promoting antibodies appear to be naturally occurring autoantibodies. Antibodies of this type are present in the serum of normal individuals and are often polyreactive IgM autoantibodies that are capable of binding to a variety of structurally unrelated, self- and non-self antigens[17]. It has been proposed that these antibodies represent a primordial form of the

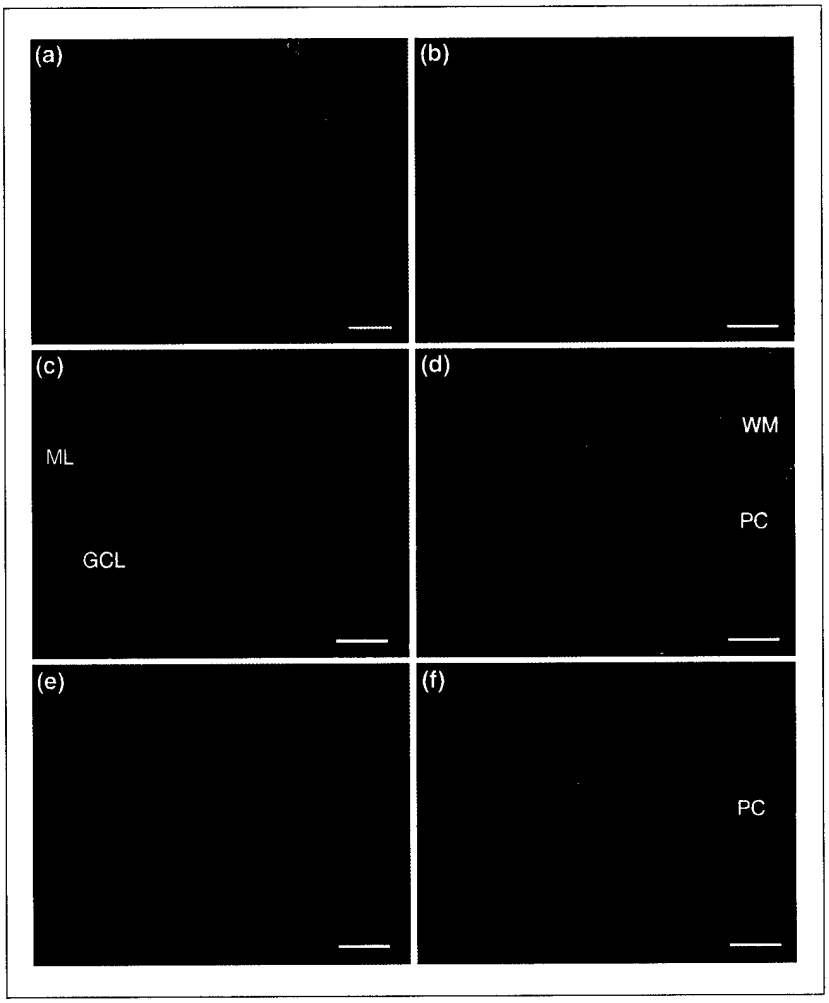

Figure 2. Natural human serum IgMs are polyreactive with a variety of cell types within the central nervous system

Slices of central nervous system tissue and cells were labeled with human antibodies before fixation or freezing to avoid artifacts. **(a)** sHIgM22 binds to the surface of oligodendrocytes obtained from the cortex of adult rats. **(b)** sHIgM42 binds to the surface of rat cerebellar granule cells after one day in culture. **(c)** Within a slice of adult SJL mouse cerebellum, AKJR4 binds strongly to the granule cell layer (GCL) and a population of basket cells within the molecular layer (ML). **(d)** sHIgM14 binds strongly to Purkinje cells (PC) and their arborizations as well as the central white matter (WM). **(e)** sHIgM32 labels the cytoskeleton of astrocytes at the surface of a slice of human cortical white matter. **(f)** In a slice of mouse cerebellum, MSI16 binds strongly to PC soma, less so to their dendritic arborizations, and does not bind to the central white matter. Scale bars are 20 μm in (a), 50 μm in (b), (e) and (f), 100 μm in (c), and 250 μm in (d).

immune system that might have performed largely physiological functions[18,19]. One of the functions of this system of antibodies could be to promote tissue repair. Whether or not this is the case, systemic administration of these antibodies to animals with myelin damage appears to have therapeutic value for enhancing repair.

The notion of an endogenous repair system is consistent with our initial observation of enhanced remyelination following immunization of experimental animals with spinal cord homogenate or individual myelin components[9]. Immunization might mimic the immune system exposure to CNS antigens that occurs after injury, resulting in an up-regulation of natural autoantibodies with CNS reactivity. We cannot say with certainty whether the specificity of the response evoked by immunization is the same as the endogenous response. However, the up-regulation of antibodies with reparative potential following immunization is clearly indicated by the fact that passive transfer of the immunoglobulin fraction from immunized animals promotes repair in the recipient[10]. In patients with MS, increases in myelin-reactive antibodies are frequently observed and might result as a reaction to exposure of CNS antigens following tissue damage.

The idea of a naturally occurring IgM-based system for tissue repair is supported by our recent observations that treatment with normal polyclonal human IgM effectively promotes remyelination, whereas the human IgG fraction is far less effective[13]. Our characterization of human mAbs from patients with monoclonal gammopathy revealed a high frequency of myelin-reactive IgM antibodies, further demonstrating that such antibodies are common in the serum of individuals with no history of neurological damage.

The binding of remyelination-promoting antibodies to the myelin-forming cells of the CNS, the presence of antibodies of this type in normal individuals, and their potential up-regulation in response to immunization with myelin or in patients with myelin damage, are all consistent with the presence of an endogenous antibody-based system of tissue repair.

Mechanisms of autoantibody-mediated remyelination

All of the remyelination-promoting antibodies bind to oligodendrocytes or myelin, and it seems reasonable to suggest that this has a direct effect on the cells being recognized. Work in other laboratories has demonstrated that oligodendrocyte-specific antibodies can cause biochemical and morphological changes in these cells. Dyer and colleagues have shown that antibodies against oligodendrocyte surface epitopes, including antibodies to GalC, sulfatide and myelin/oligodendrocyte-specific protein (MOSP), can induce changes in the organization of oligodendrocyte membrane and cytoskeletal structure[20–22]. These changes in cellular structure were preceded by antibody-induced calcium influx[23–25]. The influx of calcium might therefore play an important role in the regulation of oligodendrocyte structure and function, and could conceivably play a role in antibody-induced remyelination. Recently, our laboratory has observed similar calcium fluxes in oligodendrocytes after treatment of mixed primary glial cultures with remyelination-promoting antibodies. There appears to be a high degree of correlation between the ability of an antibody to promote

remyelination and its ability to stimulate calcium influx, suggesting a connection between these two phenomena (Allan Bieber *et al.*, unpublished observations).

Myelin-reactive autoantibodies might also work to enhance myelin repair through more indirect mechanisms. Antibody binding to damaged oligodendrocytes and myelin might stimulate repair by enhancing the opsonization and clearance of myelin debris by macrophages. The remyelination-promoting antibodies are all of the IgM isotype, and one of the properties of IgM antibodies is their efficient activation of complement. Complement is an important factor for the efficient phagocytosis of myelin by cultured macrophages, and antibody-mediated activation of complement might function to fragment myelin debris, making removal more efficient[26]. Large numbers of macrophages are often observed in demyelinated lesions, and phagocytosis of myelin debris might be an important prerequisite to efficient remyelination.

Prospects and implications

Demyelinated axons have reduced conduction velocities and are highly vulnerable to conduction block and transection. Remyelination is therefore an important therapeutic goal for the treatment of demyelinating disease. Human antibodies that promote remyelination in animal models are obvious candidates for development as reagents for the treatment of diseases such as MS.

Huang and colleagues[27] recently reported that mice pre-immunized with homologous spinal cord homogenate demonstrated enhanced axonal regeneration and functional recovery after dorsal hemisection of the spinal cord. Immunization resulted in increased levels of myelin-reactive antibodies that stimulated the outgrowth of neurites on myelin substrates. This observation was interpreted as the result of antibody-mediated blocking of myelin-associated inhibitors of axon outgrowth, an effect that might also explain the enhanced axonal regeneration and functional recovery seen *in vivo*. These experiments are reminiscent of our earlier studies in the TMEV model, in which immunization with SCH or the passive transfer of immune serum was followed by repair of damaged myelin. It is not clear whether antibodies that might block myelin-associated inhibition of axon outgrowth and those that promote remyelination have similar or completely unrelated specificities. Whichever is the case, myelin-reactive antibodies might be useful, not only to promote myelin repair following demyelinating disease, but also for the treatment of axonal damage following spinal cord injury. Such antibodies could be administered exogenously or generated *in vivo* by appropriate immunization strategies. Recent work with the IN-1 antibody, also an oligodendrocyte-reactive IgM, leads to very similar conclusions[28–30].

Many of the human mAbs that were screened for oligodendrocyte reactivity bound not only to oligodendrocytes but also to other CNS cells such as neurons. Figure 2 demonstrates the diversity of reactivity that was observed among monoclonal human IgM antibodies. Many antibodies react strongly with oligodendrocytes and CNS white matter, but many also reacted with various populations of neurons, and fewer reacted with astrocytes. The high frequency of IgMs that bind to neurons raises the possibility that these antibodies might play a role in neuronal survival and regeneration following CNS injury. Many of the neuron-reactive antibodies can serve as permissive substrates for neurite outgrowth in culture, demonstrating the potential for this type of function *in vivo* and for their use in the treatment of axonal damage (Allan Bieber *et al.*, unpublished observations).

Currently, there are few effective therapies to promote tissue repair or to prevent or reverse neurological deficits following CNS injury or disease. The characterization of endogenous immune-mediated repair mechanisms is therefore of obvious importance. An understanding of these mechanisms should open up significant new areas for the development of antibody-based therapeutics and perhaps also for small-molecule-based therapeutics and vaccines for induction of the reparative response.

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Cholesterol, Aβ and Alzheimer's disease

Tobias Hartmann

Statins have been used for many years for the treatment of hypercholesterolemia. They lower low-density lipoprotein (LDL) cholesterol, increase high-density lipoprotein (HDL) levels and are considered to be very safe. Recently, a set of potential new applications was identified for statins. In the future, these drugs could be used to treat Alzheimer's disease (AD). Past studies have suggested a link between AD and lipids and a series of reports has recently been published that significantly tightens this link and also provides some explanations at the cellular level. This review focuses on these recent developments and perspectives that appear to link cholesterol, β-amyloid and AD.

This year, research has shown that it is possible to reduce cerebral β-amyloid (Aβ) levels *in vivo* with an FDA-approved drug. This drug is called simvastin and is currently used to lower serum cholesterol[1]. Interestingly, an increase in production or deposition of Aβ at the molecular level has been correlated with increased risk of Alzheimer's disease (AD).

Genes linked to AD

Four genes have been directly linked to AD by means of point mutations that cause the disease. Whereas three of these genes are known to be involved in the molecular pathways of AD, the fourth gene differs in several aspects from the others and, until now, is the only gene known to be involved in lipid pathways. Mutations in the first three genes, amyloid precursor protein (*APP*), presenilin 1 (*PS-1*) and presenilin 2 (*PS-2*), are known to cause increased $A\beta_{42}$ production[2] (see Fig. 1 for details on the generation of Aβ). However, the toxic mechanism of Aβ action remains unknown. The fourth gene, apolipoprotein E (*apoE*), encodes a lipid-binding protein, which, among several other functions, shuttles lipids between cells[3]. Human apoE exists in three major alleles. The ε4 allele is implicated in an increased risk of hypercholesterolemia[4]. This allele exchanges lipids between cells and supports neuronal sprouting[5]. Notably, the frequency of this allele is significantly increased in AD (Ref. 6). The major difference between individuals carrying the apoE ε4 allele and those carrying APP, PS-1 or PS-2 mutations, is that carriers of the apoE ε4 allele have a good chance of escaping AD even at old age as the ε4 allele lowers the age of disease onset rather than causes the disease. Moreover, unlike the other proteins, apoE is not known to be directly involved in Aβ generation, although it could be indirectly involved. The best indication that apoE plays a role in Aβ pathology is derived from experiments using apoE transgenic or knockout mice, as they show altered Aβ depositions[7].

Epidemiology and risk factors

The role of apoE as a lipid-transport protein indicates that lipids might somehow influence Aβ production. Apart from those already mentioned, other risk factors of AD indicate the involvement of lipids. Old age is undoubtedly the most significant risk for AD, but high blood pressure as well as high cholesterol levels during mid-life moderately increases the risk of developing AD later in life[8]. This mid-life correlation is interesting because increased cholesterol levels and the beginning of Aβ depositions appear to occur in parallel. By contrast, in the clinical AD patient, serum cholesterol levels no longer correlate well with disease. In fact, cholesterol levels were found to be reduced in the brains of AD patients[9]; however, it should be noted that post-mortem of an AD brain reflects only the last stage of a progressive neurodegenerative process that started decades before. The degenerative process is very pronounced at this stage and therefore little information can be gained as to what initially started the disease process.

Recently, two retrospective epidemiological studies showed that treatment with cholesterol-lowering statins

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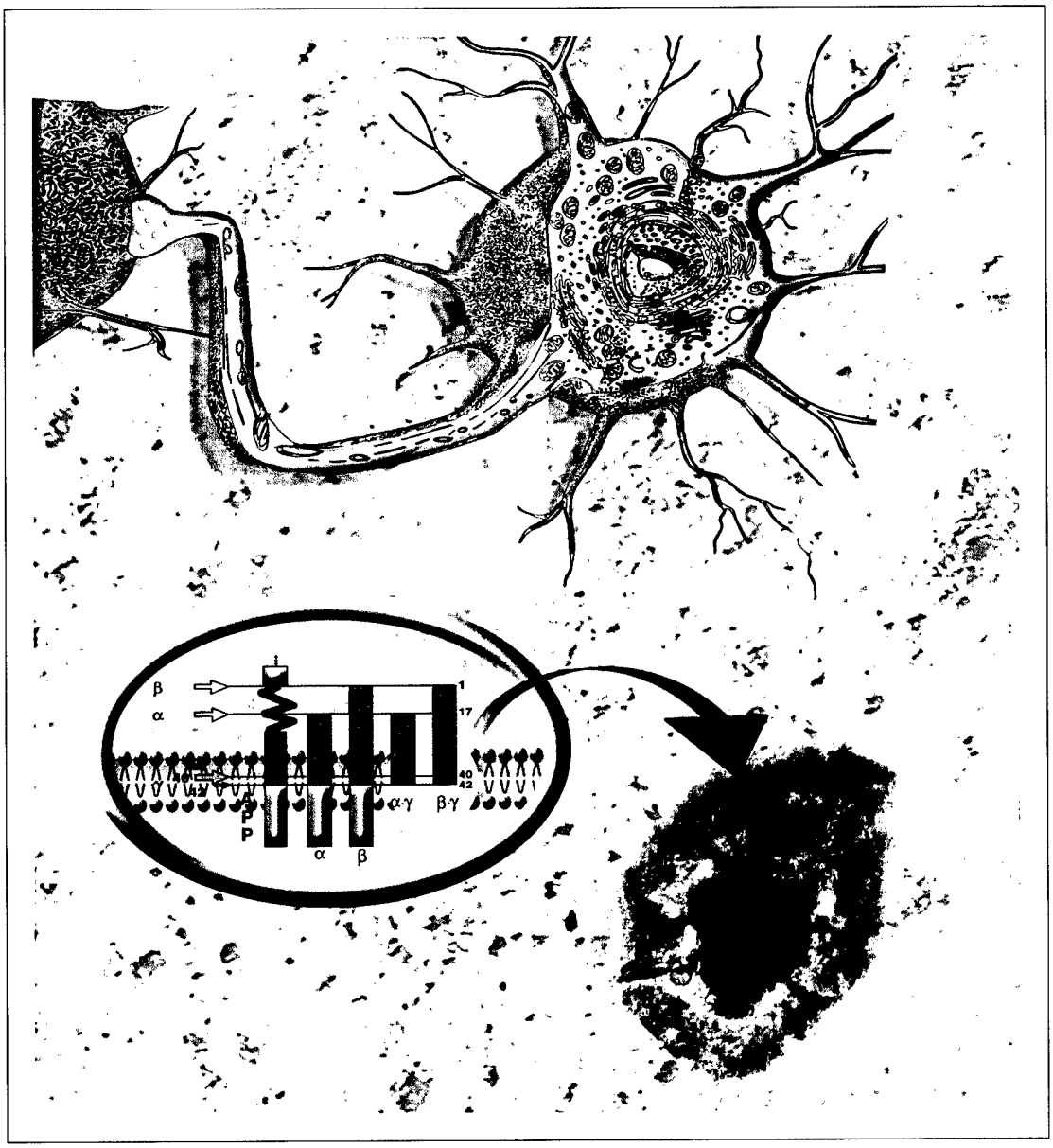

Figure 1. Aβ and APP processing

The Alzheimer human amyloid precursor protein (APP) is a 770 amino acid Type I transmembrane protein of unknown function. APP is cleaved by different proteases, termed secretases. The initial cleavage by α-secretase activity [TNFα-converting enzyme (TACE) and other proteases] is the most common cleavage, resulting in the release of the large extracellular domain of APP. Because the cleavage site of α-secretase is in the middle of the Aβ domain, no Aβ can be generated. The α-cleavage is followed by either lysosomal degradation of the remaining carboxyl-terminal fragment (C87), or by γ-secretase cleavage and the release of the small peptide p3. The γ-secretase cleavage site is located inside of the hydrophobic transmembrane region of APP, and the mechanism by which cleavage can occur at this site is unknown. The presenilins are involved in this cleavage. Less frequently, APP is cleaved by β-secretase (BACE 1). Again, this cleavage can be followed by lysosomal degradation or γ-secretase cleavage of the remaining carboxyl-terminal fragment (C99). In the latter case, Aβ is generated, which is a small 40 amino acid long, hydrophobic peptide with a tendency to aggregate and to form protease-resistant amyloid fibrils. Approximately 10% are formed with a two amino acid longer tail ($Aβ_{42}$). $Aβ_{42}$ aggregates faster, is more protease-resistant than $Aβ_{40}$, and it is $Aβ_{42}$ and not $Aβ_{40}$ that is strongly linked to familial Alzheimer's disease. Reduced cholesterol levels result in reduced β- and γ-secretase activity and increased α-secretase activity, consequently reducing Aβ levels.

drastically lowered the risk of developing AD (Refs 10,11). Statins are well-tolerated drugs[12], and could be considered as a preventive treatment for AD. The major adverse effect of statins is myopathy, which occurs in approximately 0.1% of all cases but disappears when treatment is stopped[13]. However, it should be noted that epidemiological studies cannot reveal the disease cause; this can only be achieved by experimental procedures. Although these epidemiological findings need to be interpreted with caution, it does appear that cholesterol and AD are in some way linked.

Molecular link I

How do cholesterol levels or statins influence AD etiology? To complicate this issue, statins have a variety of beneficial side-effects that might also contribute[14]. These include endothelial protection, antioxidant-, anti-inflammatory- and anti-platelet effects. The first interesting finding that suggested a link at the molecular level was obtained from rabbits, which do not normally develop Aβ deposits. Animals fed with cholesterol were found to rapidly accumulate Aβ in the brain[15]. This suggested that cholesterol affects Aβ production in a similar way to the genetic mutations in *APP*, *PS-1* and *PS-2*.

In fact, the consequence of reduced cellular cholesterol levels *in vitro* is decreased Aβ production. Primary neurons treated with statins stopped Aβ production[1,16]. Importantly, statins not only reduce total Aβ levels but also reduce $Aβ_{42}$ levels[1,17]. However, is this caused by the side-effects of statin or by lowered cholesterol levels? Statins decrease cellular cholesterol levels by inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)-reductase, whereas methyl-β-cyclodextrin (CDX) physically extracts cholesterol from the plasma membrane[18]. These are two

very different modes of action that have only two known results in common: lowered cholesterol and lowered Aβ levels[1]. It is therefore plausible that it is the reduction in cholesterol, and not the side-effects of statin, that caused decreased Aβ production in cell cultures.

It is important to consider, however, whether statins might reduce Aβ production in a less artificial situation than *in vitro* experimentation. In guinea pigs, treatment with a high dose of statins induced a sharp drop in Aβ levels in brain tissue and cerebrospinal fluid[1]. When treatment was stopped, Aβ levels were restored, indicating no permanent damage to Aβ-producing systems. However, guinea pigs do not develop amyloid plaques, irrespective of treatment. Many AD researchers suggest that amyloid plaque, and not soluble monomeric or oligomeric $A\beta_{42}$, is an important factor during early (pre-clinical) AD (Refs 19,20). Other researchers, including myself, do not share this opinion. In any case, reduced $A\beta_{42}$ levels will result in reduced or delayed plaque formation. Indeed, high cholesterol uptake increased Aβ deposition in transgenic mice[21] and rabbits[15]. A study by Refolo and colleagues[22] found that the cholesterol synthesis inhibitor BM15.766 reduced plaque formation in transgenic mice. This is a significant finding as it shows that plaque formation can actually be inhibited and that the observed effects are not drug-specific because BM15.766 acts at a later stage of cholesterol synthesis than statins. Taken together, the *in vitro* experiments suggest a tempting molecular link between AD and cholesterol, and the *in vivo* experiments not only verify this link but also provide a basis for clinical trials.

Molecular link II

Although the mechanism of cholesterol action on Aβ-producing systems remains unknown, there are interesting data that point towards effects on APP secretases. First, it was observed that inhibition of APP β-secretase occurs in parallel to cholesterol reduction[16]; and second, data were presented at the *Society for Neuroscience Conference* 2000 (8 November 2000, New Orleans, LA, USA) that showed inhibition of both APP γ-secretase and APP β-secretase by cholesterol reduction, although the precise mechanism of action apparently differs for each enzyme (Christine Bergmann and Tobias Hartmann, unpublished observations). Additional data were shown from a cell-free assay that correlated a reduction in γ-secretase activity with a reduction in membrane cholesterol content (Todd E. Golde, unpublished observations). It will be interesting to find out the extent to which lipids affect the biological function of β-site APP-cleaving enzyme (BACE) or the presenilins.

By contrast, the activity of the only remaining major secretase, non-amyloidogenic α-secretase, is increased upon cholesterol reduction, albeit under different treatment conditions[23]. Taken together, the major APP secretases are modulated in a such a way that they switch from the amyloidogenic pathway to the non-amyloidogenic pathway in response to cholesterol reduction. It is tempting to speculate that this indicates a functional link between lipids and APP processing.

The antithesis and relevance to human treatment

Although it is clear that cholesterol is able to induce the effects discussed here, the question as to whether cholesterol is directly responsible for the effects observed at the cellular level still remains to be answered. A recent report by Puglielli and colleagues indicates that similar effects could also be obtained with cholesteryl esters[24]. We know that cellular lipids are in equilibrium with each other; when cholesterol levels are depleted, its ratio to other lipids changes in parallel. Therefore, it is entirely possible that cholesterol could be an attractive target for disease treatment, but the molecule that affects secretase activity is a cholesterol derivative or some other unrelated lipid.

Remaining questions

More open questions remain unanswered. The retrospective studies reviewed here do not present data on statin dosage or duration of treatment, other than that it has to be for an extended period of time, probably many years. Because cholesterol increases and statins decrease $A\beta_{40}$- and $A\beta_{42}$-production, vascular dementia (VaD), a disease involving $A\beta_{40}$, often accompanies AD, or is interpreted as AD (Ref. 25). Whether both diseases are affected in a similar manner remains to be shown. There is also the risk of unwanted selective pressure. For example, are patients with early signs of cognitive impairment treated with statins the same as non-impaired patients? With the long pre-clinical phase of AD, it must be established at what age treatment should start. Epidemiological data show that increased serum cholesterol levels only moderately increase the risk of suffering from AD in later life. However, there could be a simple explanation to this that has broad consequences: blood cholesterol and brain cholesterol are in two separate pools. In humans, the major cholesterol source is the liver. Cholesterol is distributed from here to other organs except for the brain. The central nervous system makes up only 2% of the whole body mass, but contains approximately a quarter of the body's total unesterified cholesterol. Almost all brain cholesterol is derived from *in situ* synthesis and there is hardly any uptake from peripheral organs[26]. Therefore, epidemiological studies that measure serum cholesterol would observe only the tip of the iceberg. Furthermore, a regulatory feedback mechanism between cerebral and peripheral lipid metabolism apparently does exist. ApoE might play some role here as, for example, brain-cholesterol levels do not respond to lovastatin in apoE knock-out

mice[27]. Moreover, blood–brain barrier permeability for lipids and statins could be altered in many AD patients.

Open questions also remain from the animal experiments reviewed here. The animal data verify the principal mechanism of cholesterol affecting Aβ production *in vivo*. However, the animals were treated under different conditions from that of humans. Neither the dosage used nor the time schedule employed can be directly transferred to humans. Lower dosages and long-term treatment regimes are a must for human therapy, and the epidemiological data indicate that this is possible. Another problem is the differences between human and animal lipid metabolism; cholesterol is not a part of the normal rabbit diet, and extensive exchanges between brain and peripheral cholesterol are seen in mice. Unless the cellular mechanisms of these approaches are completely understood, it will be impossible to estimate the potential impact of new drugs.

Concluding remarks

How does lipid treatment compare with other Aβ-targeted treatment strategies? Other approaches at the top of the priority list for AD prevention are Aβ immunization and secretase inhibitors. The basic idea is identical: reduction of cerebral Aβ exposure. Because all of these treatments lower Aβ levels, one could envisage that a combination of these approaches could achieve a maximum effect with a minimum dosage of each individual drug. Eventually, all Aβ-reduction strategies will fall under the same scheme; a product of drug dosage and treatment duration started well before the onset of clinical symptoms of AD.

This will inevitably test the hypothesis that Aβ is involved in AD. Should these therapies be effective in lowering Aβ levels but ineffective in reducing the incidence of AD, it will be difficult to imagine Aβ as anything other than as a waste product of AD. Although it now appears that Aβ, Aβ-lowering strategies and AD fit neatly together, it will be the results from clinical trials that will ultimately provide the answers.

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Engineered modeling and the secrets of Parkinson's disease

Miquel Vila, Du Chu Wu and Serge Przedborski

The development of new methods for manipulating the animal genome by transgenic and gene-targeting technologies provides a unique means of studying the most intimate aspects of countless human diseases, including Parkinson's disease (PD). In this review, the contribution of such engineered models to our current understanding of the pathophysiology, etiology and pathogenesis of PD will be discussed.

Parkinson's disease (PD) is a common neurodegenerative disorder, the cardinal clinical features of which include tremor, stiffness, slowness of movement and postural instability[1]. It is estimated that, in the USA alone, more than one million individuals are currently affected by this disabling disease[1]. However, because the incidence of PD rises with age[1], it is expected that this number will increase significantly in the future because of the aging character of society. Despite this bleak perspective, several recent discoveries have unquestionably brought closer the day when the secrets of PD will be unlocked. A rapid survey of PD research not only shows the impressive pace at which advances have been made, but also that the bulk of published studies can be divided into three groups: those that investigate the pathophysiology of PD (that is, neurochemical perturbations), those that search for the etiology of PD (the cause), and those that explore the pathogenesis of PD (the mechanisms of neuronal death). This review investigates these three approaches to PD research through the appraisal of engineered models, which are, in our opinion, the spearhead of most of the recent breakthroughs accomplished in this field (Table 1). The goal of this review is to present an overview of the many recent advances in PD and not an in-depth discussion of selected topics. For further information on any of the presented subjects, the reader is encouraged to peruse the original articles referenced in this review.

Pathophysiology of PD

The main, but not sole, neurochemical alteration of PD is the deficit in brain dopamine, which is believed to be the primary culprit in the development of the motoric and non-motoric manifestations of PD (Ref. 1). This view is supported by the finding that administration of the dopamine precursor L-DOPA, which replenishes the brain with dopamine, alleviates most of the signs of PD. However, although L-DOPA is essential for the fine control of motor function, it is not necessary for the normal development of the brain circuitry within which dopamine plays such a pivotal role. Indeed, mutant mice deficient in tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, produce almost no dopamine and yet harbor a normal cytoarchitecture within the basal ganglia and the different midbrain dopaminergic neuronal groups[2]. Remarkably, pigmented $TH^{-/-}$ mice have more dopamine levels in the brain than their albino counterparts, presumably owing to the conversion of tyrosine to L-DOPA by the melanin-synthesizing enzyme tyrosinase[3]. If a similar phenomenon exists in humans then PD patients with pigmented skin or those living in sunny regions would have higher residual dopamine in the brain and thus fare clinically better than others.

Transgenic and homologous recombination technology has been extensively used to manipulate different factors in dopamine metabolism. Most of these studies have investigated the contribution of dopamine on spontaneous and drug-induced motor and non-motor behaviors. From these studies, it appears that ablation of either of the two key enzymes responsible for dopamine catabolism, namely monoamine oxidase (MAO) and catechol-O-methyl-transferase, fails to alter brain dopamine levels or locomotor activity, although all of the mutant mice studied were found to exhibit increased aggressive behavior[4,5]. Similarly, mice heterozygous for vesicular monoamine transport-2 (VMAT-2), which is the brain

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Table 1. Selected engineered models used in the study of Parkinson's disease[a]

Category	Refs
Pathophysiology	
Tyrosine hydroxylase$^{-/-}$ mice	2,3
Monoamino oxidase$^{-/-}$ mice	4
Catechol-O-methyl-transferase$^{-/-}$ mice	5
Vesicular monoamine transporter$^{+/-}$ mice	6
Vesicular monoamine transporter transfected cells	7
Dopamine transporter$^{-/-}$ mice	8
Dopamine receptor$^{-/-}$ mice	9
Etiology	
α-Synuclein$^{-/-}$ mice	12
α-Synuclein transgenic (wild-type, A53T, A30P)	13–18
Pathogenesis	
Oxidative stress	
Cu–Zn-superoxide dismutase$^{-/-}$ mice	26
Manganese-superoxide dismutase$^{+/-}$ mice	28
Glutathione peroxidase$^{-/-}$ mice	26
Monoamino-oxidase B transgenic	29
Trophic factor support	
GDNF$^{-/-}$ mice	30
BDNF$^{-/-}$ mice	31
TGFα$^{-/-}$ mice	34
Mitochondria	
Adenine nucleotide translocator$^{-/-}$ mice	35
Complex I 'cybrids'	36

[a]Abbreviations: BDNF, brain-derived neurotrophic factor; GDNF, glial-derived neurotrophic factor; TGF, transforming growth factor.

carrier that enables translocation of cytosolic monoamines into synaptic vesicles, also show minimal baseline abnormalities[6]. However, as predicted, VMAT-2$^{+/-}$ mice exhibit diminished extracellular striatal dopamine levels, as well as reduced K^+- and amphetamine-evoked dopamine release[6]. Conversely, VMAT-2 overexpression in small synaptic vesicles of transfected dopaminergic neurons shows increased quantal size and frequency of dopamine release consistent with the recruitment of synaptic vesicles, which do not normally release dopamine[7]. Together, these studies demonstrate that VMAT-2 is a critical regulator of the rate of transmitter accumulation and synaptic strength in the monoamine system. Mutant mice deficient in dopamine transporter (DAT) are spontaneously much more metabolically perturbed in that, by virtue of lacking the ability to recapture released dopamine, they exhibit a dramatic increase in striatal extracellular dopamine levels and are already grossly hyperactive at baseline[8]. This abnormal motor behavior is probably related to a hyperstimulation of the postsynaptic dopaminergic receptors in response to the higher extracellular content of dopamine. If this interpretation is correct, then it also explains why mutant mice deficient in at least dopamine D2 receptors have an impaired capacity for responding to extracellular dopamine and why, as a consequence, are so hypoactive[9]. As expected, this poor locomotor activity could not be improved by the administration of dopamine agonist[9]. In agreement with our current knowledge of the distribution and function of the minor dopamine receptors, manipulation of D3-, D4- and D5-receptor expression demonstrates that they all seem to exert opposing motoric effects to those of the D2 receptor[9].

The aforementioned studies indicate that many tools are already available to target key components of the dopamine system, including its synthesis and degradation, its intracellular compartmentalization, its release, and its postsynaptic neurotransmission. So far, published data demonstrate that, unless conspicuous changes in striatal dopamine or in dopamine postsynaptic neurotransmission occurs, no gross motor abnormalities arise in these engineered animals. This conclusion is consistent with the finding that parkinsonism emerges in humans only after severe alterations in dopaminergic pathways[1].

Etiology of PD

The cellular basis of PD is a dramatic loss of dopaminergic neurons, primarily in the substantia nigra pars compacta (SNpc)[1]. The cause of PD neurodegeneration remains unknown. However, the discovery that rare familial forms of PD are linked to genetic mutations has raised the prospect that, through the study of these unique pedigrees, some hints into the etiology of PD can be obtained. So far, five disease-causing loci and mutations in three genes, as well as several allelic associations, have been linked to PD[1]. Mutant proteins derived from causal mutations include: (1) α-synuclein, which is linked to an autosomal dominant form of PD; (2) parkin, which is linked to an autosomal recessive form of early-onset PD; and (3) ubiquitin carboxy-terminus hydrolase L1 (UCH-L1), which is linked to an autosomal dominant form of typical PD. Other mutations have been identified in association with clinical syndromes that comprise parkinsonism, such as point mutations in the Tau gene[10],

Table 2. Transgenic α-synuclein animals[a]

Species	Form of α-synuclein	Promoter	Loss of SNpc DA cells	Striatal DA deficit	Inclusions	Motor deficits	Refs
Mouse	Wild-type	PDGF	No	Yes	Nuclear and cytoplasmic, no fibrillar aggregates, in neocortex, hippocampus and, 'occasionally' in SN	Yes	13
Mouse	Wild-type and mutant (A53T)	Thy-1	No	No	Lewy-like pathology, especially in motor neurons	Yes	14
Mouse	Mutant (A30P)	Thy-1 and TH	No	No	Somal and neuritic accumulation of mutant α-synuclein	No	15
Mouse	Wild-type and mutant (A53T, A30P)	TH	No	No	No	No	16
Mouse	Wild-type and mutant (A30P)	Thy-1	No	No	Abnormal accumulation of α-synuclein in cell bodies and neurites	No	17
Drosophila	Wild-type and mutant (A53T, A30P)	GAL4	Yes	Yes	Yes	Yes	18

[a]Abbreviations: DA, dopamine; PDGF, platelet-derived growth factor; SN, substantia nigra; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase

which in humans causes a form of frontotemporal dementia with parkinsonism.

α-Synuclein and PD

To date, two PD-causing missense mutations in α-synuclein (A53T and A30P) have been identified[1]. Cytotoxicity of mutant α-synuclein is probably related to the fact that both identified point mutations might enhance the propensity of this small presynaptic protein to interact with other proteins and aggregate[11] to form Lewy-body-like intraneuronal inclusions, a pathologic hallmark of PD (Ref. 1). The lack of α-synuclein results in neither the parkinsonian phenotype nor alterations in dopaminergic pathways in mice[12]. Still, these mutant animals exhibit increased dopamine release following paired stimuli, a mild reduction in striatal dopamine content and an attenuation of dopamine-dependent locomotor response to amphetamine, suggesting that α-synuclein negatively regulates dopamine neurotransmission. More complicated is the situation of transgenic animals overexpressing either wild-type or mutant α-synuclein, which have generated inconsistent results (Table 2). For instance, overexpression of wild-type α-synuclein driven by platelet-derived growth factor promoter is associated with the accumulation of α-synuclein- and ubiquitin-immunoreactive inclusions reminiscent of Lewy bodies in the neocortex, hippocampus and occasionally in the substantia nigra[13]. Despite an absence of nigral dopamine-mediated neuronal loss, aged transgenic animals show a reduction in striatal TH protein content and enzymatic activity that ascribes to impaired motor performance. In a second line of transgenic mice overexpressing either the wild-type or mutant allele, α-synuclein-containing inclusions with some neuronal death were found in the spinal cord but not in the SNpc, where the promoter used is not expressed[14]. And, in three additional lines of transgenic animals in which expression of wild-type or mutant α-synuclein is driven by either a neuronal glycoprotein Thy-1 promoter or a TH promoter that enables transgene expression in SNpc, no nigrostriatal pathology was found at all[15–17]. Unlike the mouse, expression of wild-type or mutant α-synuclein in fruit flies reproduces most of the features of PD, including a selective age-dependent loss of dorsomedial dopaminergic neurons, Lewy-body-like α-synuclein-positive inclusions and a progressive age-dependent locomotor dysfunction[18]. Because α-synuclein can be damaged by oxidative stress[19,20], it is worth mentioning that fruit flies have a more intense oxidative metabolism than mice. Thus, it is possible that the apparent discrepancy between the inconsistent and subtle abnormalities found in transgenic α-synuclein mice and the robust alterations found in transgenic α-synuclein *Drosophila* is related to a different degree of post-translational modification of α-synuclein inflicted by oxidative stress.

Ubiquitin metabolism-linked mutations and familial parkinsonism

As indicated previously, another PD-causing mutation (I93M) is found in the gene encoding UCH-L1, a key

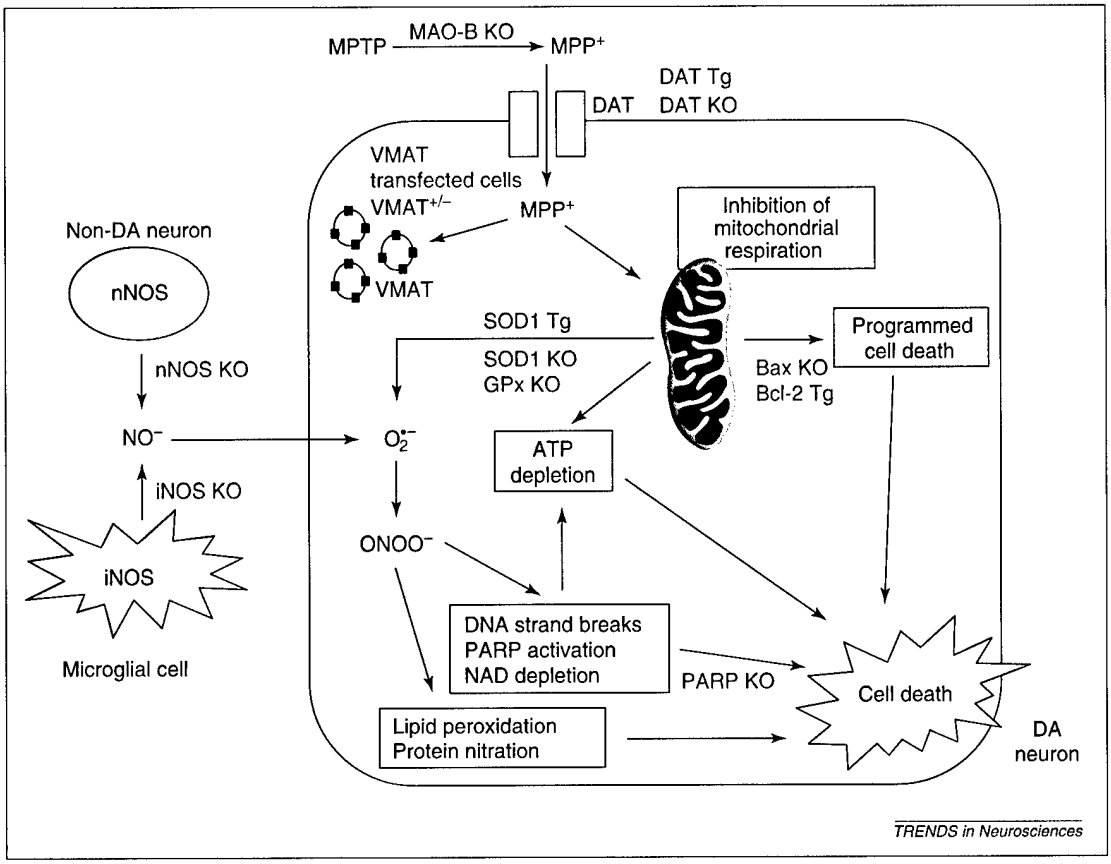

Figure 1. Engineered manipulations of MPTP molecular mechanism

Engineered animals (knockouts, KO, or transgenics, Tg) enable targeting of specific molecular factors hypothesized to be instrumental in the demise of dopaminergic neurons induced by the parkinsonian neurotoxin MPTP. Intervention aimed at blocking neurotoxic effects of MPTP is shown in red. Intervention aimed at enhancing the neurotoxic effects of MPTP is shown in green. Further information regarding the use of engineered animals in the MPTP model can be found in Ref. 24. Abbreviations: DAT, dopamine transporter; GPx, glutathione peroxidase; iNOS, inducible nitric oxide synthase; MAO-B, monoamino oxidase B; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; nNOS, neuronal nitric oxide synthase; PARP, poly(ADP-ribose) polymerase; SOD1, copper–zinc superoxide dismutase; VMAT, vesicular monoamine transporter.

enzyme in the ubiquitin pathway[21]. This is an exciting finding in light of the fact that ubiquitin is highly expressed in Lewy bodies and that parkin, which, upon mutation, causes a juvenile form of PD, possesses a ubiquitin-related activity[22]. Although, so far, no engineered animals exist for either UCH-L1 or parkin mutations, it has been established that the gracile axonal dystrophy (*gad*) mutant mouse carries a spontaneous autosomal recessive mutation resulting in an in-frame deletion of *UCH-L1*[23]. These mutant mice do not show any clinical or pathological similarity to PD patients harboring UCH-L1 mutation, but they represent the first mammalian model of neurodegeneration with a defect in the ubiquitin system.

Pathogenesis of PD

Following the initiation of the disease by an etiological factor, mounting evidence indicates that a cascade of deleterious events is set in motion, which will ultimately be responsible for the demise of dopaminergic neurons. Over the years, several pathogenic hypotheses have been proposed and, with the development of engineered animals, these are now testable. Investigations geared toward studying the pathogenesis of PD can be divided into two broad categories: (1) those in which engineered animals are used directly to test hypothesized pathogenic mechanisms; and (2) those in which engineered animals are used to modulate the susceptibility of dopaminergic neurons to a given insult, such as the parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)[24] or rotenone[25].

Engineered animals used to test PD pathogenic mechanisms directly

The most popular pathogenic hypotheses for PD include oxidative stress (related to dopamine metabolism or other mechanisms), inappropriate trophic support, reactivation of apoptosis, excitotoxicity, and mitochondrial dysfunction. In keeping with these dominant themes, it is

relevant to mention that mice lacking various key scavenging enzymes for reactive oxygen species (ROS) do not show overt SNpc neurodegeneration[24,26–28]. By contrast, transgenic mice overexpressing the dopamine-metabolizing enzyme MAO-B show no actual neuronal death but do exhibit a striking atrophy of SNpc neurons[29], probably caused by increased ROS production during dopamine deamination. Even more dramatic is the effect of the lack of trophic factors. First, mutant mice deficient in glial-derived neurotrophic factor (GDNF) or brain-derived neurotrophic factor (BDNF) do not survive beyond postnatal day 21 (Refs 30 and 31). $GDNF^{-/-}$ neonates suffer from major abnormalities in both peripheral and central noradrenergic neurons, although midbrain dopamine neurons appear intact[30]. This observation is quite surprising given the importance of GDNF in dopaminergic neuron survival[32] and the intense developmental SNpc neuronal death seen in the mouse[33], a phenomenon thought to be highly sensitive to trophic support. More in line with this latter argument is the fact that mutant mice deficient in transforming growth factor-α have a significantly lower number of SNpc dopaminergic neurons than their wild-type littermates[34]; but here, surprisingly, the other midbrain dopaminergic neuronal populations appear unaffected by the lack of this trophic factor[34].

In connection with the proposed role of mitochondria as the main source of ROS responsible for the oxidative attack in PD, it has been demonstrated that oxidative phosphorylation can be inhibited by ablating the adenine nucleotide translocator (Ant-1), thus resulting in widespread oxidative damage in these mice[35]. However, contrary to this hypothesis, the brunt of Ant-1 deficit was found in muscles and not in the SNpc (or in the brain as a whole)[35]. Furthermore, in cells, transgenicity (cytoplasmic hybrids or 'cybrids') has been used to evaluate the consequences of the defect in complex I in PD (Ref. 36). In this study, cybrid cells with reduced complex I activity exhibit a variety of major functional alterations, which all have potential pathogenic significance and could easily, either separately or in combination, kill SNpc dopaminergic neurons. Finally, there are several mouse models with SNpc alterations that occur for unclear reasons. These include: (1) transgenic mice expressing a mutant form of the enzyme superoxide dismutase-1 (SOD1), which not only show a loss of spinal cord motor neurons but also a reduction in the number of SNpc dopaminergic neurons[37]; (2) mice lacking the orphan nuclear receptor Nurr-1, which fail to generate midbrain dopaminergic neurons and are hypoactive[38]; (3) mutant mice lacking engrailed genes En-1 and En-2, showing that these genes control the survival of midbrain dopaminergic neurons in a dose-dependent manner and regulate the expression of α-synuclein[39]; (4) mice knockout for the estrogen receptor-β, which exhibit several morphological brain abnormalities and a pronounced neuronal degeneration with aging, particularly in the SNpc[40]; and (5) mice deficient in the ATM gene (known to be involved in DNA repair), and which also develop severe degeneration of SNpc dopaminergic neurons[41].

Engineered animals used to modulate the susceptibility of dopaminergic neurons to a given insult

To date, several potent neurotoxins have been used to duplicate most of the biochemical and neuropathological hallmarks of PD. Worth noting, however, is the fact that the chronic infusion of the mitochondrial poison rotenone into rats seems to be the only toxin-induced animal model of PD that is unequivocal in showing Lewy-body-like intraneuronal inclusions[25]. So far, however, rotenone-induced SNpc toxicity has only been documented in rats[25] and, to our knowledge, has yet to be used in engineered animal models, which precludes its discussion here. The situation is quite different for MPTP, which has been used in transgenic and knockout mice[24] and which has contributed significantly to our current understanding of the pathogenesis of PD. After the first wave of fruitful studies in the 1980s led to the characterization of the key steps in MPTP metabolism, this neurotoxin had a second wave of interest with the advent of engineered animals[24]. This powerful combination has provided a well-recognized and validated tool to not only injure, specifically, dopaminergic neurons but also to target specific molecular factors hypothesized to be instrumental in the demise of these neurons (Fig. 1). The benefit of this dual approach has definitively confirmed the nature of those factors that determine the neurotoxic potency of MPTP (Ref. 24). For instance, it has been shown that mutant mice deficient in MAO-B fail to transform the pro-toxin MPTP into its active metabolite 1-methyl-4-phenylpyridinium (MPP^+) and consequently are refractory to the deleterious effect of this toxin[42]. Mutant mice deficient in DAT are also resistant to MPTP[43], thus confirming the mandatory role of DAT in MPP^+ entry into dopaminergic neurons and the ensuing cytotoxicity. As for VMAT-2, which enables MPP^+ sequestration into synaptic vesicles, engineered mice have shown that the lower the VMAT-2 expression, the greater the MPTP-induced dopaminergic toxicity[44]. This shows how crucial cytosolic MPP^+ is to the MPTP neurotoxic process.

This combined approach has also shed light on the molecular mechanisms that could be of importance to

unraveling the pathogenesis of PD (Fig. 1). It is well established that once MPP^+ is in the dopaminergic neuron, it is actively concentrated into the mitochondria where, through its binding to complex I, it interrupts the flow of electrons, which leads to a deficit in ATP formation and to increased production of ROS, especially superoxide radical[24]. The importance of the latter in the neurotoxic process of MPTP is supported by the finding that transgenic mice with increased activity of SOD1, the key protective enzyme against superoxide, are more resistant to MPTP than their non-transgenic littermates, whereas mutant mice deficient in SOD1 or in glutathione peroxidase are more sensitive[24,26]. However, it is known that superoxide, which is poorly reactive, is unlikely to be the sole mediator of oxidative damage inflicted on dopaminergic neurons following MPTP administration. To circumvent this issue, it has been proposed that superoxide reacts with nitric oxide (NO), a small molecule present in great abundance in the brain, to produce the highly reactive tissue-damaging species, peroxynitrite. Relevant to the hypothesized involvement of peroxynitrite in MPTP-induced neuronal death are the observations that mutant mice deficient in either neuronal or inducible NO synthase are partially protected against MPTP (Refs 24 and 27) by depleting NO in the brain. Another consequence of MPTP attack or intoxication is the activation of the apoptotic cascade, an active form of cell death, presumably implicated in the overall neurodegenerative process in PD. So far, supportive evidence for this pathogenic mechanism is provided by the finding that ablation of the pro-apoptotic protein Bax in mutant mice[45] or, conversely, overexpression of the anti-apoptotic protein Bcl-2 in transgenic mice, attenuates dopaminergic neuronal death caused by MPTP (Ref. 24).

Concluding remarks

In this review, we have tried to compile the most recent and significant reports dealing with the complex issue of how to better understand and treat PD. Using the angle of engineered models, we have shown how eclectic and important the advances accomplished in the field of PD research are. From this, it clearly emerges that the contribution of transgenic and knockout animals has been, and will continue to be, tremendous as far as the study of the pathogenesis of PD is concerned. To date, comparable strides have not been achieved in the study of the etiology of PD but, with several PD-linked gene mutations still to be tested using this technology, it is our opinion that brighter days are ahead and major breakthroughs, which could change the landscape of PD, are just around the corner.

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Antisense as a neuroscience tool and therapeutic agent

Peter Estibeiro and Jenny Godfray

Gene expression in the mammalian brain is highly complex and requires an immensely powerful functional genomics tool to unravel it. Antisense has the potential to meet this requirement, but has always been plagued by biological and technological hurdles that have made the technology unreliable. With recent progress in developing potent, low-toxicity nucleic acid chemistries and novel drug delivery methods to cross the blood–brain barrier, the use of antisense is gathering momentum.

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Imagine a technology that enables you to reach into a specific cell type in the brain and to switch off the expression of a single gene product precisely. Think of the power of such a technology as a functional genomics tool or as a rational therapy. This is the promise of antisense; but since its conceptualization in the 1970s it has never quite lived up to its potential. The antisense research community has risen to the challenge and many of the early problems associated with the technology have been addressed. Antisense has been given another chance and the question now is whether it can deliver its promise.

The antisense concept is simple and elegant. Gene expression relies on genomic DNA sequences being transcribed into mRNA that are, in turn, translated into proteins. An antisense molecule is a complementary nucleic acid that hybridizes specifically to its mRNA target, preventing its translation. Any existing protein encoded by the target gene depletes as a function of its half-life, and thus a rapid and highly specific 'knockdown' is achieved. Given suitable model systems, antisense is the ideal tool for gene function studies. Its main advantages over transgenic-based germline knockouts are speed and cost. In addition, acute changes to gene expression can be made in a developmentally normal system, enabling interpretation in the absence of developmental redundancy and compensation: factors that are crucial to neuroscientists asking what a gene product does in a fully developed adult brain.

Antisense can also be used to dissect disease pathways in model systems and to identify steps amenable to therapeutic intervention: a process called target validation. Unlike the conventional process, a validating antisense is itself a lead candidate for further development as a drug, thus eliminating any requirement for screening banks of molecules for effect against the target. Antisense can further shortcut the drug discovery process because all antisense reagents are chemically the same; they differ only in their sequence. This simplifies manufacturing set-up, and aspects of drug trials relating to chemical toxicity and nonspecific effects need be carried out in full only once on a generic control reagent.

Turning the antisense concept into reality has not been simple. Many obstacles have been encountered (Table 1), but progress is being made in overcoming them. Two related technologies are shown in Boxes 1 and 2.

Mechanisms of action

To work effectively as an antisense reagent, a synthetic oligonucleotide must be relatively stable when introduced into a biological system. Unmodified nucleic acids are readily broken down by nucleases omnipresent in blood, cells and, to a lesser extent, cerebrospinal fluid, within a few hours of injection into an animal. To extend the half-life sufficiently for antisense molecules to elicit an effect, the oligonucleotide chemistry must be modified to be less susceptible to nuclease attack.

Whenever novel nucleic acid chemistries are employed as antisense reagents, consideration of the mechanisms that underlie antisense knockdown is needed. There is evidence for two distinct processes[1] (as illustrated in Fig. 1). The primary mechanism relies on antisense-mediated degradation of the transcript by endogenous RNase H. Antisense chemistries that do not support RNase H activity rely instead on physical interference with translation[2]. Ideally, nucleotide analogues used as antisense reagents

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Table 1. Technological hurdles faced in the development and application of antisense technology

Technological hurdle	Comments
Stability	Unmodified nucleic acids have a half-life of only a few minutes when introduced into biological systems. They are rapidly broken down by endogenous nucleases and are thus unsuitable for use in most antisense experiments. Stability has been addressed by using modified nucleic acid analogues and, more recently, peptide nucleic acids.
Toxicity	Some antisense reagents are toxic at relatively modest concentrations, and a fine balance is thus required between administering enough of the reagent to elicit an antisense effect and not so much as to elicit nonspecific chemical toxicity.
Delivery	A means of delivering the antisense reagent to its target biological system and then into cells is required. The use of antisense as a neuroscience tool and a therapeutic agent has an extra dimension of difficulty: the reagent must be delivered across the blood–brain barrier.
Sequence targeting	mRNA has complex higher order structures and is best thought of as a tangle of string. Over 90% of any mRNA is prevented by intra-molecular interactions from hybridizing to an antisense molecule. The key to successful antisense design is to identify those sequences in the target mRNA that are accessible to pair with the bases of the introduced antisense molecule.
Specificity	Theoretically, antisense-mediated gene knockdown should be very selective and very specific. However, nonspecific biological effects are sometimes seen, and designing the correct controls is crucial to the success of an antisense project, particularly in neuroscience, where the assay might detect subtle behavioural changes.
Immune response	Although a perceived hurdle to the use of antisense, there is little evidence that the immune system mounts a significant response to exogenous nucleic acids or nucleic acid analogues. The immune system does respond strongly to nucleic acids containing unmethylated CpG dinucleotides, which are seen as bacterial and therefore hostile (CpG effect)[44]. In most, if not all, cases where an immune response has been mounted against an antisense oligonucleotide, it can be attributed to the CpG effect.

should be non-toxic, readily soluble and taken up by their target cells. They should have strong hybrid affinity with their target mRNA and should support both suggested antisense mechanisms.

Methylphosphonate oligodeoxynucleotides (Fig. 2b) were among the first modified oligonucleotides to be tested as antisense reagents[3]. These are highly resistant to nuclease degradation but do not support RNase H activity and, being uncharged, are not readily soluble. Solubility is restored by substituting the methyl groups for sulfur to create phosphorothioate oligodeoxynucleotides (S-ODN; Fig. 2c). S-ODNs are still comparatively nuclease resistant and are highly soluble in aqueous fluids; importantly, they readily support RNase H activity. However, they do not bind their target mRNAs as tightly as unmodified oligonucleotides and have to be used at high concentrations (several μM) to compensate.

Toxicity

Although S-ODNs have been the mainstay of antisense research for many years, they are largely responsible for the reputation of antisense as producing nonspecific toxicity. S-ODNs are highly polyanionic and can bind heparin-binding proteins; they can, for example, displace basic fibroblast growth factor from its low-affinity receptors and can prevent epidermal growth factor (EGF) from binding to its receptor while promoting auto-phosphorylation of the EGF receptor in the absence of EGF. (Potential pitfalls of using S-ODNs as antisense molecules are reviewed in detail in Ref. 4.)

S-ODN toxicity has been investigated in both primates and rodents[5]. At concentrations of up to 5 mg kg^{-1}, well above those currently used for antisense research or clinical trials, and over a six-month period, the S-ODN becomes most concentrated in the kidneys and liver, causing quite serious but reversible anomalies and some immune stimulation. Primates are much less affected than rodents but neither shows any evidence of teratogenic effects, or of changes in reproductive performance or fertility. This study did not directly address the use of S-ODNs in CNS. Test S-ODNs were administered systemically and it is doubtful whether they would have crossed the blood–brain barrier to any great extent. When S-ODNs are injected directly into the brain, weight loss[6] or severe tissue damage[7,8] has been reported.

Chemistry

One way of addressing S-ODN toxicity is to increase their affinity for their specific mRNA targets. Modifications to the 2′-sugar position can give rise to significant increases in duplex stability and therefore enable the oligonucleotides to be used at lower concentrations; however, they do not support RNase H activity[9]. The G-clamp heterocycle modification, a cytosine analogue that clamps onto guanine by forming an additional hydrogen bond, was

Box 1. Ribozymes

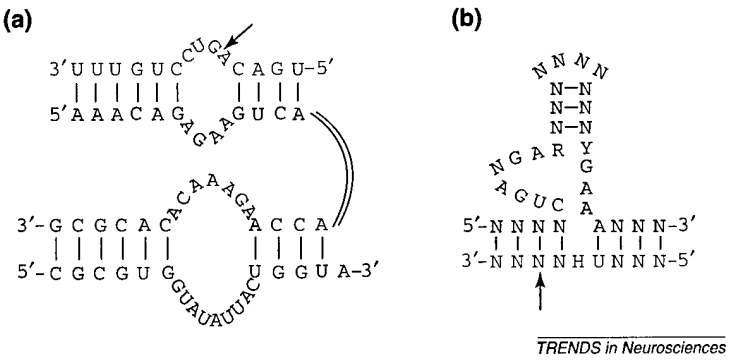

Figure I. Ribozyme structures

Interaction between **(a)** hairpin and **(b)** hammerhead ribozymes and their target RNAs (blue). The arrow indicates the position of the cleavage site. H is any nucleotide except G; N is any nucleotide; R is a purine nucleotide; Y is a pyrimidine nucleotide.

Ribozymes are RNA molecules that are able to cleave phosphodiester bonds without the aid of protein-based enzymes. Hammerhead and hairpin ribozymes are sequence specific (Fig. I), and both have been used as antisense molecules (structure and mechanism of action are reviewed in Ref. a). Ribozymes can be delivered *in vivo* directly as synthetic oligonucleotides or expressed from transgenes. A variety of chemical modifications have been tested to stabilize the molecule, optimize delivery, increase RNA hybrid strength and improve cleavage efficiency[b,c]. Ribozymes selected empirically are more successful than those designed by computer modelling[d].

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Box 2. RNA interference

RNA interference (RNAi) technology can also silence gene expression. Double-stranded RNA (dsRNA), which is homologous to the target transcript, is introduced into cells where it is broken down into short interfering (si) RNAs (also double stranded) that trigger the degradation of mRNAs with complementary sequences. Although used successfully in *Caenorhabditis elegans* and *Drosophila*, dsRNA in mammalian cells leads to a global shutdown in protein synthesis[a]. Recent work[b] that has gone some way to overcoming these difficulties might lead to more widespread use of RNAi as a method for controlling mammalian gene expression.

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rationally designed to enhance oligonucleotide–RNA hybrid affinity. G-clamp S-ODNs are about 25-fold more potent than equivalent S-ODNs (Ref. 10). Little has been done to assess the nonspecific effects of these chemistries at therapeutic concentrations, but fewer problems are reported. Chimeric oligonucleotides in which phosphorothioates protect more vulnerable phosphodiesters from nuclease attack also help to reduce nonspecific effects. These molecules, particularly when additionally modified by partial 2′-methoxylation (to enhance duplex stability) elicit antisense effects after intraventricular administration without the toxicity associated with equivalent S-ODN controls[6].

Novel oligonucleotide backbones are continually being developed to further enhance antisense potency while eliminating toxicity. Prominent among the new generation chemistries are N3′–P5′phosphoramidate (NP)[11], morpholino phosphorodiamidate (MF)[12], locked nucleic acid (LNA)[8], 2′-O-methoxyethyl (MOE)[2] and 2′-fluoro, arabino-nucleic acid (FANA)[13] (Fig. 2d–h). All are highly resistant to nuclease attack and have very high affinity for their target. FANA and LNA oligonucleotides support RNase H activity but NP, MF and MOE oligonucleotides do not. Most interesting of the new chemistries from a neuroscience point of view are LNAs. These are specific and potent when injected into rat CNS, with no reported toxicity[8]. No rodent toxicity has been reported for NP or MF oligonucleotides either, although currently CNS-specific toxicology has not been studied.

An alternative to backbone modifications is to replace the phosphodiester backbone completely, as in peptide nucleic acids (PNA; Fig. 2i)[14,15]. Because they are uncharged, PNAs form very stable duplexes with DNA or RNA. Unfortunately, this also means that they are not taken up efficiently by cells. They also do not support RNase H activity. Cell uptake has been addressed by conjugating the PNA to molecules such as anti-receptor antibodies that aid cellular internalization. (PNAs as antisense tools are reviewed in Ref. 16.) RNase H support can be restored by covalently linking PNA to DNA (Ref. 17): a short phosphodiester window within a PNA will evoke RNase H activity against target mRNA (Ref. 18). These chimeric DNA/PNA oligos are very readily taken up by cells, enabling PNAs to be administered at micromolar concentrations. Some cytotoxicity has been reported at very high PNA concentrations (reviewed in Ref. 19).

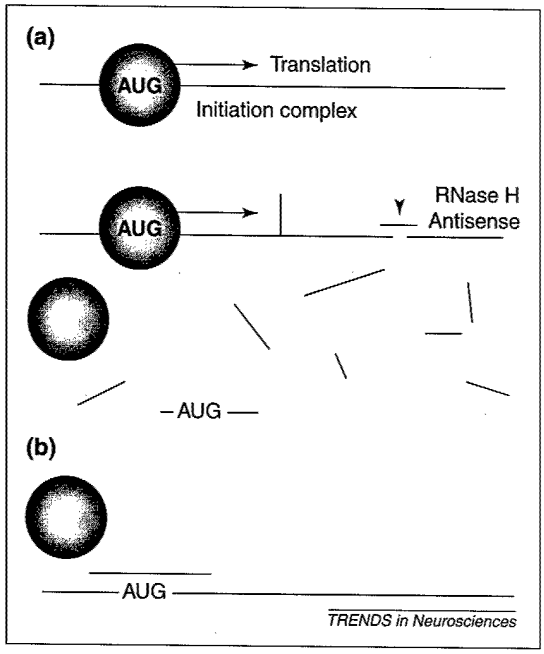

Figure 1. Mechanisms by which antisense oligonucleotides disrupt mRNA function

(a) Top: translation initiation complex docks with AUG start site on mRNA. Middle: antisense oligomer hybridizes with mRNA. RNase H recognizes the RNA–DNA heteroduplex, degrades the RNA strand and translation is inhibited. Bottom: cleaved mRNA is rapidly degraded further by other intracellular ribonucleases and is thus not available for translation. **(b)** A second mechanism of action suggests that rather than tagging the mRNA for degradation, the bound antisense molecule physically interferes with translation. In this case, the level of the target protein, but not the mRNA, is depleted.

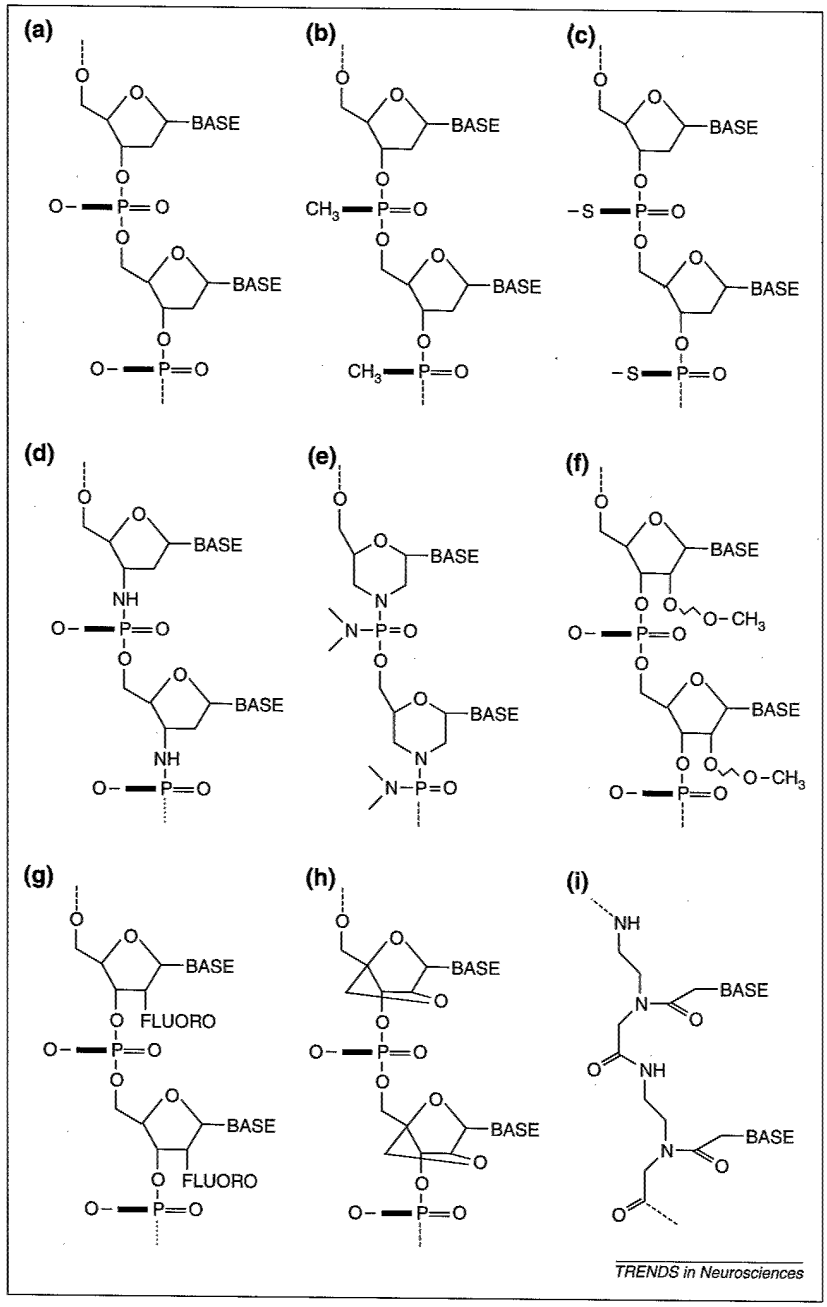

Figure 2. Candidate antisense oligomers

Chemical structures of candidate oligomers for use in antisense research and therapies. Structural differences to the unmodified phosphodiester oligonucleotide **(a)** are shown in **(b)–(h)**. (a) Unmodified phosphodiester oligonucleotide. (b) Methylphosphonate oligonucleotide (a methyl group replaces on the non-bridging oxygen atoms at each phosphorous in the ribose phosphate backbone). (c) Phosphothioate oligonucleotide (sulfur replaces the methyl group of a methylphosphonate oligonucleotide). (d) N3′-P5′ phosphoramidate oligonucleotide (contains N3′-P5′ phosphoramidate linkages). (e) Morpholino phosphorodiamidate oligonucleotide (six-membered morpholine backbone moieties joined by non-ionic phosphorodiamidate intersubunit linkages). (f) 2′-O-methoxyethyl oligonucleotide (substitution of a methoxyethyl side chain on the 2′-sugar position). (g) 2′-fluoro,arabino-nucleic acid (2′ stereoisomer of RNA based on D-arabinose instead of the natural D-ribose, containing a 2′ fluoro group). (h) Locked nucleic acid (the ribose ring is constrained by a methylene linkage between the 2′-oxygen and the 4′-carbon). **(i)** Peptide nucleic acid (contains a non-charged achiral polyamide backbone).

Targeting antisense to mRNA sequences

The vast majority of any transcript is involved in or screened by intramolecular base-pairing, and is unavailable for antisense interaction[20]. The key to successful antisense design, therefore, is to identify accessible areas of a transcript and to use this knowledge to target antisense molecules against it rationally.

Although there is some evidence for sequence motifs correlating with effective antisense reagents[21], it is still necessary to map transcript structure in detail to determine accessibility to antisense. Two technologies currently used to determine mRNA structure are computational algorithms and laboratory-based experimental mapping. Both methods correlate for short transcript lengths, but algorithms cannot reliably predict structure when transcripts exceed 200 nucleotides. The average transcript is over 1 kb, and given that distal sequences can contribute to proximal structure, computational analysis is currently unreliable for high fidelity antisense design. (Computational methods are reviewed in Ref. 22.) New algorithms based on probability profile might have advantages over minimum free energy calculations[23], but it is too early to comment on the real predictive power of this technique. Thus, experimental mapping is currently the preferred rational antisense design tool. It is an excellent predictor of sequences that will make effective antisense targets *in vivo*[24].

Table 2. Some examples of antisense technology applied to neuroscience research

Target mRNA	Chemistry	Delivery	Controls	Conclusions drawn from study	Refs
Rat δ-opioid receptor	Phosphorothioate	Injection or infusion into lateral ventricles	Mis-matched antisense sequence	Knockdown of the δ-opioid receptor reduces binding of receptor ligands to brain membranes. The δ-opioid receptor agonist SNC80 has no effect on knockdown rats, indicating that SNC80, when administered systemically, acts in the brain to affect locomotor behaviour and colonic propulsion. Response to morphine is unchanged in knockdowns, which confirms existing germline knockout data that morphine-induced analgesia is modulated by the μ-opioid receptors.	34
Rat μ-opioid receptor	Peptide nucleic acid	Intraperitoneal injection	Mis-match sequences and an antisense targeted to another receptor	Significant decrease in μ-opioid receptor levels in the periaqueductal grey region causes a reduction in the antinociceptive response to morphine. This result supports existing germline knockout studies and is complementary to the δ-opioid receptor knockdown experiment outlined above.	37
Rat glial glutamate transporter (GLT1)	Phosphorothioate	Infusion into lateral ventricles	Sense and random sequences	GLT1 promotes neuronal survival after traumatic brain injury in rats by 'mopping up' released glutamate and preventing receptor over-activation.	38
Rat GLT1 as above and neuronal glutamate transporter (EAAC1)	Phosphorothioate	Infusion into lateral ventricles	Sense and random sequences	GLT1 promotes neuronal survival after focal cerebral ischaemia in rats by 'mopping up' released glutamate and preventing receptor over-activation. EAAC1 does not.	39
Mouse $GABA_B$ receptor gb1a	Phosphorothioate	Mouse intermediate lobe tumour cells explanted into culture in the presence of antisense reagents	Scrambled antisense sequence and antisense designed to $GABA_B$ receptor gb1b	The activity of the GABA-receptor agonist gabapentain is dependent on a functional $GABA_B$ (gb1a-gb2) heterodimer receptor.	40
Mouse amyloid precursor protein (APP)	Phosphorothioate	Intra-cerebroventricular injection	Random sequence and ineffective antisense sequences	Antisense used together with antibodies against target protein have an additive effect and significantly reverse learning and memory deficits in the aged SAMP8 mouse model for Alzheimer's disease.	41
Rat high-affinity nerve growth factor (NGF) receptor (trkA)	Phosphorothioate	Stereotaxic injection into medial septal area or cornu ammonis (CA1) of hippocampus	Random sequence	Data indicate a role for NGF acting on choline acetyl-transferase and vesicular acetylcholine transporter via the trkA receptor in contextual memory consolidation.	42
Mouse presenilin 1	Phosphorothioate	Cortical neurons explanted into culture and exposed to antisense reagents	Sense, scrambled and mis-matched antisense sequences	Reduction in endogenous presenilin 1 expression results in protection against NMDA-induced cell death. Suggests that familial Alzheimer's disease-associated presenilin 1 variants might increase the vulnerability of neurons to excitotoxicity. These results support existing data obtained from germline knockouts.	43

The basis for current experimental methods is that mRNA structures can be mapped by hybridizing a folded *in vitro* transcript with a complementary library of overlapping oligonucleotides. Care must be taken to ensure the transcript folds as it transcribes and is not subsequently denatured. Cleavage of duplexes with RNase H (Ref. 25) or gel retardation of labelled oligonucleotides after transcript binding[26] can form the basis of an assay to determine which of the oligonucleotides are able to bind the transcript, thus indicating accessible regions.

The most significant recent advance in mapping RNA structures is antisense oligonucleotide (AS-ON) scanning microarrays, as pioneered by Southern[27,28]. By covalently attaching all AS-ONs to be tested to a solid support, their interaction with a labelled target transcript applied to the array under hybridizing conditions can be analysed. Those

immobilized AS-ONs that have the strongest interactions with the target transcript have a high probability of effectively knocking down gene expression *in vivo*.

Delivery of antisense oligonucleotides to their site of action

To elicit an effect, antisense reagents must be delivered into target cells at the required site of action. In neuroscience, the blood–brain barrier must also be overcome. S-ODNs are easily taken up by cells and localize to the nucleus, which might be their site of action[29]. Cell types take up S-ODNs with differing efficiencies; neurons are more efficient at taking up S-ODNs than other types of brain cell (Peter Estibeiro, unpublished observation). LNA (Ref. 8) and MF (Ref. 30) oligonucleotides are also taken up by cells without the need for artificial transfection aids, and MF and NP (Ref. 11) oligonucleotides show nuclear localization. To improve cell uptake, oligonucleotides can be complexed with cationic lipid or polyamine carriers. A wealth of literature exists on antisense transfection[31,32].

PNAs are uncharged and cannot theoretically cross either the blood–brain barrier or the plasma membrane unaided. However at high (millimolar) concentrations, PNAs injected systemically can knockdown expression of specific genes in the brain[33,34]. PNA delivery is enhanced by conjugation to peptides that facilitate transport across the blood–brain barrier and into cells, for example, the OX26 mAb to the rat transferrin receptor[35]. Once inside cells, at least a proportion of PNAs reach the nucleus[36].

Most administration of antisense into the brain has been by direct injection or infusion, thus physically crossing the blood–brain barrier. Although this is an acceptable means of delivery in humans for the treatment of serious conditions such as brain tumours, it is clear that a less invasive method is necessary for antisense to realize its potential as a therapeutic agent for the CNS. Conjugating oligonucleotides or PNAs to carriers has huge potential in this regard and will surely be a rapidly developing neuroscience technology over the next few years.

Antisense in the nervous system: progress and conclusions

The use of antisense in neuroscience research and therapeutics is gathering momentum. The toxicity issues associated with S-ODNs are being addressed through the use of current-generation nucleic acid analogues. LNAs and PNAs are reported not to be neurotoxic. Neither has any rodent toxicity been reported after systemic administration of NPs and MFs, but so far these have not yet been tested in the brain. Current chemistries have minimal non-antisense effects, making design of controls and interpretation of results easier, particularly in neuroscience (where assays might be subtle). In a therapeutic context, low-toxicity, high-specificity reagents will minimize side-effects and enable the use of higher drug doses.

Care must still be taken over controls. Sense strand is often used but is not ideal: it can interfere with natural antisense effects. Similarly, random sequence controls should be avoided. The best control is scrambled experimental sequence because it maintains chemical identity with the test reagent. Using a variety of backbone chemistries also provides an excellent control: an identical result from two different backbones implies a genuine antisense effect.

Crossing the blood–brain barrier remains a challenge. The brute force method of injecting reagents into the brain is successful, and several studies of neurodegeneration and brain-function research, some examples of which are outlined in Table 2, are emerging[34,37–43]. At least one pharmaceutical company has an antisense-based anti-Alzheimer's disease drug in trials, which might be administered by an implanted infusion device, indicating that invasive delivery will be tolerated for human CNS therapy if warranted by the disease. In the longer term, conjugation of antisense reagents to small molecules that carry them across the blood–brain barrier and into specific cell types is the way forward, and pioneering work in this area looks very promising[35].

The future for antisense looks brighter than ever. It is probably the most powerful functional genomics tool currently available to meet the challenge of understanding the genome; and it is finally coming of age as a rational therapeutic agent.

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Blockade of Microglial Activation Is Neuroprotective in the 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine Mouse Model of Parkinson Disease

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1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) damages the nigrostriatal dopaminergic pathway as seen in Parkinson's disease (PD), a common neurodegenerative disorder with no effective protective treatment. Consistent with a role of glial cells in PD neurodegeneration, here we show that minocycline, an approved tetracycline derivative that inhibits microglial activation independently of its antimicrobial properties, mitigates both the demise of nigrostriatal dopaminergic neurons and the formation of nitrotyrosine produced by MPTP. In addition, we show that minocycline not only prevents MPTP-induced activation of microglia but also the formation of mature interleukin-1β and the activation of NADPH–oxidase and inducible nitric oxide synthase (iNOS), three key microglial-derived cytotoxic mediators. Previously, we demonstrated that ablation of iNOS attenuates MPTP-induced neurotoxicity. Now, we demonstrate that iNOS is not the only microglial-related culprit implicated in MPTP-induced toxicity because mutant iNOS-deficient mice treated with minocycline are more resistant to this neurotoxin than iNOS-deficient mice not treated with minocycline. This study demonstrates that microglial-related inflammatory events play a significant role in the MPTP neurotoxic process and suggests that minocycline may be a valuable neuroprotective agent for the treatment of PD.

Key words: IL-1β; iNOS; minocycline; microglia; MPTP; NADPH–oxidase; neurodegeneration; Parkinson's disease

Parkinson's disease (PD) is a common neurodegenerative disorder whose cardinal clinical features include tremor, slowness of movement, stiffness, and postural instability (Fahn and Przedborski, 2000). These symptoms are primarily attributable to the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the consequent loss of their projecting nerve fibers in the striatum (Hornykiewicz and Kish, 1987; Pakkenberg et al., 1991). Although several approved drugs do alleviate PD symptoms, chronic use of these drugs is often associated with debilitating side effects (Kostic et al., 1991), and none seems to dampen the progression of the disease. So far, the development of effective neuroprotective therapies is impeded by our limited knowledge of the pathogenesis of PD. However, significant insights into the mechanisms by which SNpc dopaminergic neurons may die in PD have been achieved by the use of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which replicates in humans and nonhuman primates a severe and irreversible PD-like syndrome (Przedborski et al., 2000). In several mammalian species, MPTP reproduces most of the biochemical and pathological hallmarks of PD, including the dramatic neurodegeneration of the nigrostriatal dopaminergic pathway (Przedborski et al., 2000).

To elucidate PD pathogenic factors, and thus to develop therapeutic strategies aimed at halting its progression, we revisited the neuropathology of this disease in search of putative culprits. Aside from the dramatic loss of dopaminergic neurons, it appears that the SNpc is also the site of a robust glial reaction in PD and experimental models of PD (Vila et al., 2001b). Although gliosis and especially activated microglia may sometimes be associated with beneficial effects, often gliosis appears to be deleterious (Vila et al., 2001b). For instance, microglial cells, which are resident macrophages in the brain, have the ability to react promptly in response to insults of various natures (Kreutzberg, 1996) in that resting microglia quickly proliferate, become hypertrophic, and increase or express *de novo* a plethora of marker molecules (Banati et al., 1993; Kreutzberg, 1996). The multifunctional nature of activated microglia encompasses the upregulation of cell surface markers such as the macrophage antigen complex-1 (MAC-1), phagocytosis, and the production of cytotoxic molecules, including reactive oxygen species (ROS), nitric oxide (NO), and a variety of proinflammatory cytokines such as interleukin-1β (IL-1β) (Banati et al., 1993; Gehrmann et al., 1995; Hopkins and Rothwell, 1995). Given this, there is little doubt that activated microglia, through the actions of aforementioned factors, can inflict significant damage on neighboring cells.

Minocycline, a semisynthetic second-generation tetracycline, is an antibiotic that possesses superior penetration through the

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brain–blood barrier (Aronson, 1980). Minocycline has emerged as a potent inhibitor of microglial activation (Amin et al., 1996; Yrjanheikki et al., 1998, 1999; Tikka and Koistinaho, 2001; Tikka et al., 2001a), an anti-inflammatory property completely separate from its antimicrobial action, and as an effective neuroprotective agent in experimental brain ischemia (Yrjanheikki et al., 1998, 1999), in the R6/2 mouse model of Huntington's disease (Chen et al., 2000), in traumatic brain injury (Sanchez Mejia et al., 2001), and in the 6-hydroxydopamine model of PD (He et al., 2001). In the present study, we report that, in the MPTP mouse model of PD, minocycline (1) mitigates, in a dose-dependent manner, the loss of dopaminergic cell bodies in the SNpc and of nerve terminals in the striatum, (2) reduces the levels of nitrotyrosine, a marker of protein nitrative modification, (3) prevents microglial activation with minimal effects on the astrocytic response, (4) reduces the formation of mature IL-1β and decreases activation of NADPH–oxidase and upregulation of inducible nitric oxide synthase (iNOS), two enzymes implicated in microglial-derived production of ROS and NO, respectively, and (5) protects against MPTP beyond the beneficial effect of iNOS ablation (Liberatore et al., 1999; Dehmer et al., 2000).

MATERIALS AND METHODS

Animals and treatment. All mice used in this study were 8-week-old male C57BL/6 mice from Charles River Laboratories (Wilmington, MA) and iNOS-deficient mice (C57BL/6-NOS2; The Jackson Laboratory, Bar Harbor, ME) and their wild-type littermates weighing 22–25 gm. For MPTP intoxication, mice received four intraperitoneal injections of MPTP-HCl (18 or 16 mg/kg of free base; Sigma, St. Louis, MO) in saline at 2 hr intervals. For minocycline treatment, mice received twice daily (12 hr apart) intraperitoneal injections of varying doses of minocycline-HCl ranging from 1.4 to 45 mg/kg (Sigma) in saline starting 30 min after the first MPTP injection and continuing through 4 additional days after the last injection of MPTP; control mice received saline only. Mice (n = 5–8 per group; saline–saline, saline–minocycline, MPTP–saline, and MPTP–minocycline) were killed at selected time points, and their brains were used for morphological and biochemical analyses. Procedures using laboratory animals were in accordance with the National Institutes of Health guidelines for the use of live animals and were approved by the institutional animal care and use committee of Columbia University. MPTP handling and safety measures were in accordance with our published recommendations (Przedborski et al., 2001b).

Immunoblots. Cytosolic and particulate fractions from selected mouse brain regions were prepared as described previously (Vila et al., 2001a) and used for either one-dimensional Western blot or dot-blot analyses. For Western blots, the following primary antibodies were used: monoclonal anti-p67phox (1:1000; Transduction Laboratories, Lexington, KY), polyclonal anti-calnexin (1:2000; Stressgen, Victoria, British Columbia, Canada). For dot-blot analyses, 25 μg of protein extracts were loaded onto the 0.2 μm nitrocellulose membrane in dot-blot apparatus (Bio-Rad, Hercules, CA), and blots were probed with an affinity-purified polyclonal antibody against nitrotyrosine (1:1000) (Przedborski et al., 2001a) that was preconjugated overnight at 4°C with 1:5000 dilution of horseradish-labeled donkey anti-rabbit IgG. For all blots, bound primary antibody was detected using a horseradish-conjugated antibody against IgG and a chemiluminescent substract (SuperSignal Ultra; Pierce, Rockford, IL). All films were quantified using the NIH Image analysis system.

RNA extraction and reverse transcription-PCR. Total RNA was extracted from midbrain, striatal, and cerebellar samples from all four groups of mice at selected time points and used for reverse transcription-PCR analysis as described previously (Vila et al., 2001a). The primer sequences used in this study were as follows: for mouse MAC-1, 5′-CAG ATC AAC AAT GTG ACC GTA TGG-3′ (forward) and 5′-CAT CAT GTC CTT GTA CTG CCG C-3′ (reverse); for mouse glial fibrillary acidic protein (GFAP), 5′-CAG GCA ATC TGT TAC ACT TG-3′ (forward) and 5′-ATA GCA CCA GGT GCT TGA AC-3′ (reverse); and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-GTT TCT TAC TCC TTG GAG GCC AT-3′ (forward) and 5′-TGA TGA CAT CAA GAA GTG GTG AA-3′ (reverse). PCR amplification was performed for 26 cycles for MAC-1 and GFAP and 18 cycles for GADPH. After amplification, products were separated on a 5% PAGE. Gels were dried and exposed overnight to a phosphorimager screen, and then radioactivity was quantified using a computerized analysis system (Bio-Rad PhosphoImager system).

Immunohistochemistry and stereology. Brains were fixed and processed for immunostaining as described previously (Liberatore et al., 1999). Primary antibodies used in this study were as follows: rat anti-MAC-1 (1:200; Serotec, Raleigh, NC), mouse anti-GFAP (1:1000; Boehringer Mannheim, Indianapolis, IN), and a rabbit polyclonal anti-tyrosine hydroxylase (TH) (1:1000; Calbiochem, San Diego, CA). Immunostaining was visualized by using either 3,3′-diaminobenzine (brown) or SG substrate kit (gray blue; Vector Laboratories, Burlingame, CA). Sections were counterstained with thionin.

The total number of TH-positive SNpc neurons was counted in the various groups of animals at 7 d after the last MPTP or saline injection using the optical fractionator method as described previously (Liberatore et al., 1999). This is an unbiased method of cell counting that is not affected by either the volume of reference (SNpc) or the size of the counted elements (neurons). Striatal density of TH immunoreactivity was determined as described previously (Burke et al., 1990).

Assay of NOS catalytic activity. Ventral midbrain NOS activity was assessed by measuring both the calcium-dependent and calcium-independent conversion of [^{3}H]arginine to [^{3}H]citrulline as described previously (Liberatore et al., 1999).

Mature IL-1β measurement. Ventral midbrain content of mature murine IL-1β was done as described using an enzyme-linked immunosorbend assay kit specific for this cytokine (R & D Systems, Minneapolis, MN) (Li et al., 2000).

Measurement of striatal levels of 1-methyl-4-phenylpyridinium. This was done in MPTP–saline and MPTP–minocycline mice killed at 90 min after one intraperitoneal injection of 18 mg/kg MPTP using an HPLC method with ultraviolet detection (wavelength, 295 nm) as described previously (Przedborski et al., 1996).

Synaptosomal 1-methyl-4-phenylpyridinium uptake. Naïve mice were killed, and their striata were dissected out and processed for uptake experiments as described previously (Przedborski et al., 1992). The uptake of [^{3}H]1-methyl-4-phenylpyridinium (MPP^+) was assessed in the absence and presence of minocycline (concentration raging from 1 to 330 μM). The assay was repeated three times, each time using duplicate samples.

Mouse tissue slices and lactate measurement. Striatal slices (300 μm) were prepared and processed as described by Kindt et al. (1987) using 50 μM MPP^+ and varying concentrations of minocycline (0–333 μM). At the end of the incubation (60 min; 37°C), media were collected and used for lactate quantification by enzymatic assay based on the formation of NADH, followed by 340 nm in a spectrophotometer. The assay was repeated three times, each time using duplicate samples.

Statistical analysis. All values are expressed as the mean ± SEM. Differences between means were analyzed using a two-tail Student's t test. Differences among means were analyzed using one-way ANOVA, with time, treatment, or genotype as the independent factors. When ANOVA showed significant differences, pairwise comparisons between means were tested by Newman–Keuls *post hoc* testing. In all analyses, the null hypothesis was rejected at the 0.05 level.

RESULTS

Minocycline attenuates MPTP-induced dopaminergic neurodegeneration

As illustrated in Figure 1*G*, the numbers of SNpc TH-positive neurons varied significantly among the various groups of mice ($F_{(9,71)} = 7.045$; $p < 0.001$). MPTP, 18 mg/kg for four injections over 8 hr, caused more than a 55% reduction in the number of SNpc dopaminergic neuron numbers, as evidenced by TH immunostaining (Fig. 1*C*,*G*). In MPTP-treated mice, minocycline increased significantly the number of surviving SNpc TH-positive neurons in a dose-dependent manner (Fig. 1*D*,*G*). Minocycline at a dose of 1.4 mg/kg twice daily had no effect on MPTP neuro-

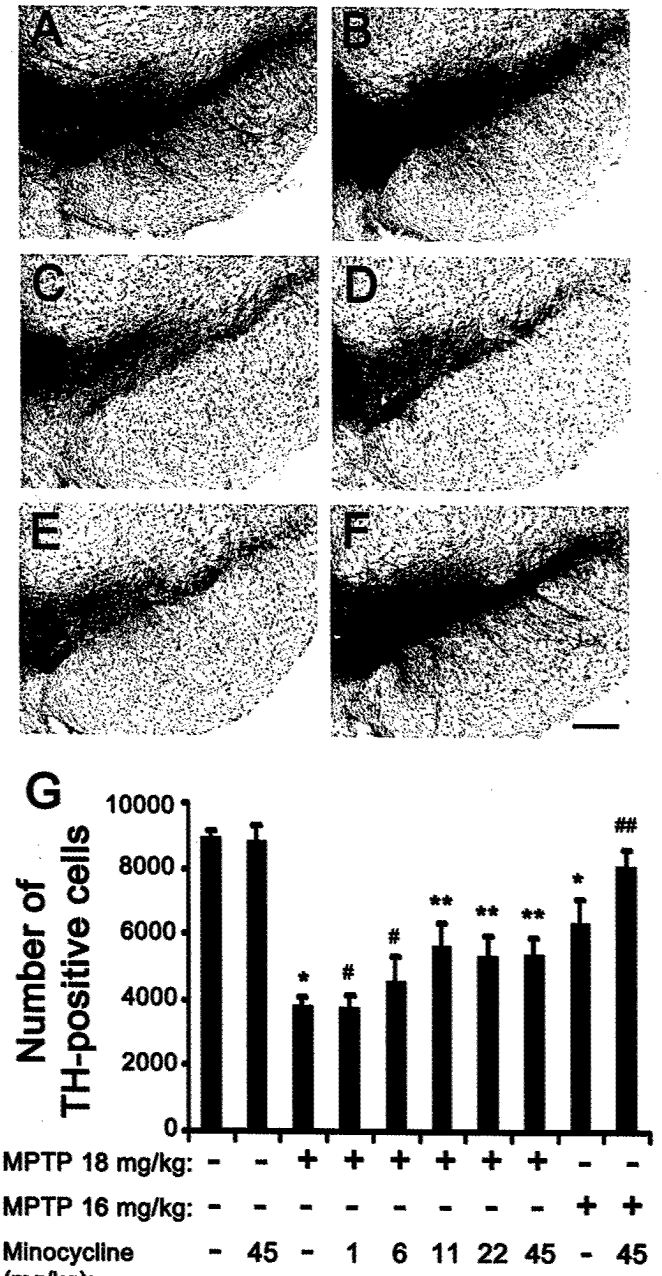

Figure 1. Effect of minocycline on MPTP-induced SNpc dopaminergic neuronal death. In saline-injected control mice treated without (*A*) or with (*B*; 45 mg/kg twice daily) minocycline, there are numerous SNpc TH-positive neurons (*brown*; *A*, *B*). MPTP (18 mg/kg for 4 injections) reduces the number of SNpc TH-positive neurons (*C*) 7 d after the last injection. In mice treated with both MPTP and minocycline, there is a noticeable attenuation of SNpc TH-positive neuronal loss (*D*). At a lower MPTP dosage (16 mg/kg for 4 injections), loss of TH-positive structures is less (*E*) and minocycline protection is more obvious (*F*). Scale bar, 50 μm. Bar graph shows SNpc TH-positive neuronal counts (*G*) assessed under the various experimental conditions. *Minocycline 1, 6, 11, 22, 45*, Mice injected with minocycline at 1.4, 6.1, 11.3, 22.5, and 45.0 mg/kg twice daily. $*p < 0.05$, fewer than saline-injected or minocycline-injected control mice. $\#p > 0.05$, same as MPTP-injected mice. $**p < 0.05$, fewer than control mice but more than MPTP-injected mice. $\#\#p < 0.05$, more than MPTP-injected mice and not different from control mice. Values are means ± SEM (n = 6–8 per group).

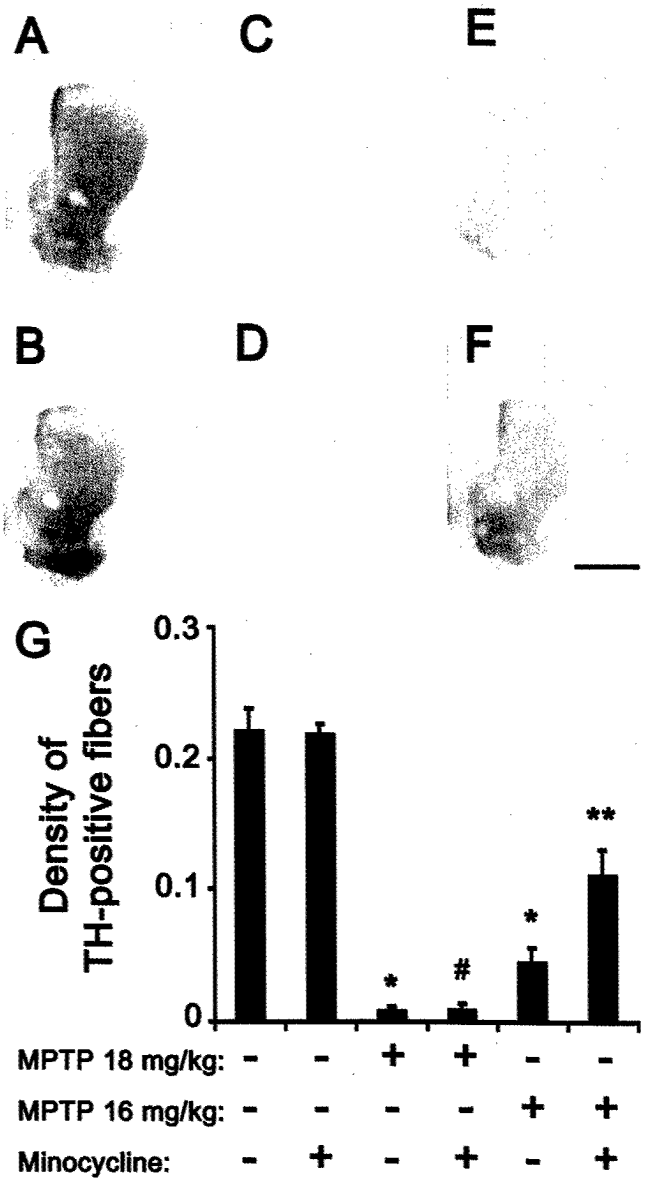

Figure 2. Effect of minocycline on MPTP-induced striatal dopaminergic fiber loss. In saline-injected control mice treated without (*A*) or with (*B*; 45 mg/kg twice daily) minocycline, there are a high density of striatal TH-positive fibers. MPTP (18 mg/kg for 4 injections) reduces the density of striatal TH-positive fibers (*C*) 7 d after the last injection. In mice treated with both MPTP and minocycline, there is also a noticeable striatal TH-positive fiber loss (*D*). At a lower MPTP dosage (16 mg/kg for 4 injections), loss of TH-positive structures is less (*E*) and minocycline protection is obvious (*F*). Scale bar, 1 mm. Bar graph shows striatal TH-positive optical density (*G*) assessed under the various experimental conditions ($F_{(5,33)} = 41.475$; $p < 0.001$). $*p < 0.05$, fewer than saline-injected or minocycline-injected control mice. $\#p > 0.05$, same as MPTP-injected mice. $**p < 0.05$, more than MPTP-injected mice but fewer than control mice. Values are means ± SEM (n = 6–8 per group).

toxicity, whereas at doses of 11.25 mg/kg twice daily and higher, there was significant neuroprotection (Fig. 1*G*). Even at the highest dose tested (45 mg/kg twice daily), minocycline was well tolerated and did not produce any behavioral abnormality. To test whether minocycline could provide complete neuroprotection, we examined another group of mice with less severe SNpc damage by

Table 1. Nitrotyrosine levels (pg/μg protein)

	Saline	Minocycline	MPTP	MPTP–minocycline
Ventral midbrain	16.2 ± 1.3	18.5 ± 1.7	32.2 ± 6.0*	21.8 ± 1.8**
Cerebellum	13.1 ± 0.8	14.0 ± 2.1	13.4 ± 1.0	11.7 ± 1.1

Nitrotyrosine levels are significantly different among groups in the ventral midbrain ($F_{(3,23)} = 4.56; p < 0.05$) but not in cerebellum ($F_{(3,23)} = 0.618; p > 0.05$). *$p < 0.05$, more than saline-injected and minocycline-injected control mice. **$p < 0.05$, less than MPTP-injected mice but not different from both control groups. Saline, Mice injected with saline; Minocycline, mice injected with minocycline only (45 mg/kg twice daily); MPTP, mice injected with MPTP only (18 mg/kg MPTP for 4 injections in one day); MPTP–minocycline, mice injected with both MPTP and minocycline. Values are means ± SEM (n = 6–8 per group).

Table 2. Striatal MPTP metabolism

MPP^+ level				
Treatment	MPTP only	MPTP–minocycline pretreatment	MPTP–minocycline post-treatment	
μg/gm tissue	6.42 ± 0.92	5.21 ± 0.66	6.52 ± 0.59	
[^{3}H]MPP^+ uptake				
Minocycline (μM)	0	10	100	333
% of control	100	98 ± 3	96 ± 3	82 ± 1
MPP^+-induced lactate				
Minocycline (μM)	0	10	100	333
μM/100 mg protein	74 ± 4	71 ± 6	70 ± 6	67 ± 6

For MPP^+ levels, minocycline (45 mg/kg) was given either 30 min before or after MPTP administration. Values are means ± SEM of either six mice per group (MPP^+ levels) or three independent experiments each performed in duplicate ([^{3}H]MPP^+ uptake and lactate levels). None of the presented values differ significantly ($p > 0.05$) from MPTP only (MPP^+ levels) or from 0 μM minocycline ([^{3}H]MPP^+ uptake and lactate levels).

injecting a lower dose of MPTP (16 mg/kg for four injections). In mice that received MPTP only, this lower regimen reduced numbers of SNpc TH-positive neurons by ~30% compared with controls (Fig. 1*E*,*G*). Minocycline at 45 mg/kg twice daily produced >90% protection against MPTP at 16 mg/kg for four injections (Fig. 1*F*,*G*).

Sparing of SNpc dopaminergic neurons does not always correlate with sparing of their corresponding striatal nerve fibers (Liberatore et al., 1999), which is essential for maintaining dopaminergic neurotransmission. To determine whether minocycline can prevent not only MPTP-induced loss of SNpc neurons but also the loss of striatal dopaminergic fibers, we assessed the density of TH immunoreactivity in striata from the different groups of mice (Fig. 2). Four injections of MPTP at 18 and 16 mg/kg reduced striatal TH immunoreactivity compared with controls by 96 and 79%, respectively (Fig. 2*C*,*E*,*G*). Mice that received minocycline (45 mg/kg twice daily) and four injections of 18 mg/kg MPTP (Fig. 2*D*,*G*) showed no protection of striatal dopaminergic fibers, whereas mice that received the same dose of minocycline and four injections of 16 mg/kg MPTP (Fig. 2*F*,*G*) showed significant sparing of striatal TH-positive fibers. These findings indicate that minocycline protects the nigrostriatal pathway against the effects of the parkinsonian toxin MPTP.

Minocycline decreases MPTP-mediated nitrotyrosine formation

A significant part of the MPTP neurotoxic process is mediated by NO-related oxidative damage (Przedborski et al., 2000), the extent of which can be evaluated by assessing nitrotyrosine levels (Liberatore et al., 1999; Pennathur et al., 1999). In saline-injected mice, the levels of nitrotyrosine in ventral midbrain were similar between non-minocycline and minocycline-treated animals (Table 1). In MPTP-injected mice (18 mg/kg for four injections), nitrotyrosine levels were significantly increased in ventral midbrain (brain region containing SNpc) and unchanged in cerebellum (brain region unaffected by MPTP) (Table 1). MPTP

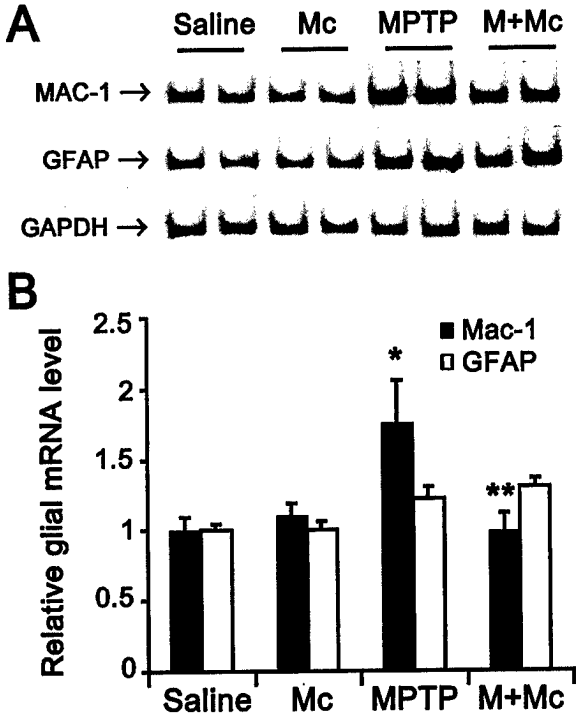

Figure 3. Minocycline prevents MPTP-induced MAC-1 transcription. *A*, *B*, Ventral midbrain MAC-1 mRNA levels but not GFAP mRNA levels are increased by 24 hr after MPTP injection compared with those of saline- or minocycline-injected mice. Minocycline prevents MPTP-induced MAC-1 mRNA increases. MAC-1 and GFAP mRNA values are normalized with GAPDH. Values are mean ± SEM ratios (n = 5–7 mice per group). *Saline*, Saline-treated; *Mc*, minocycline-treated; *MPTP*, MPTP-treated; *M + Mc*, MPTP plus minocycline-treated. *$p < 0.05$, higher than both saline- and minocycline-injected control groups. **$p < 0.05$, lower than MPTP-injected group and not different from both control groups.

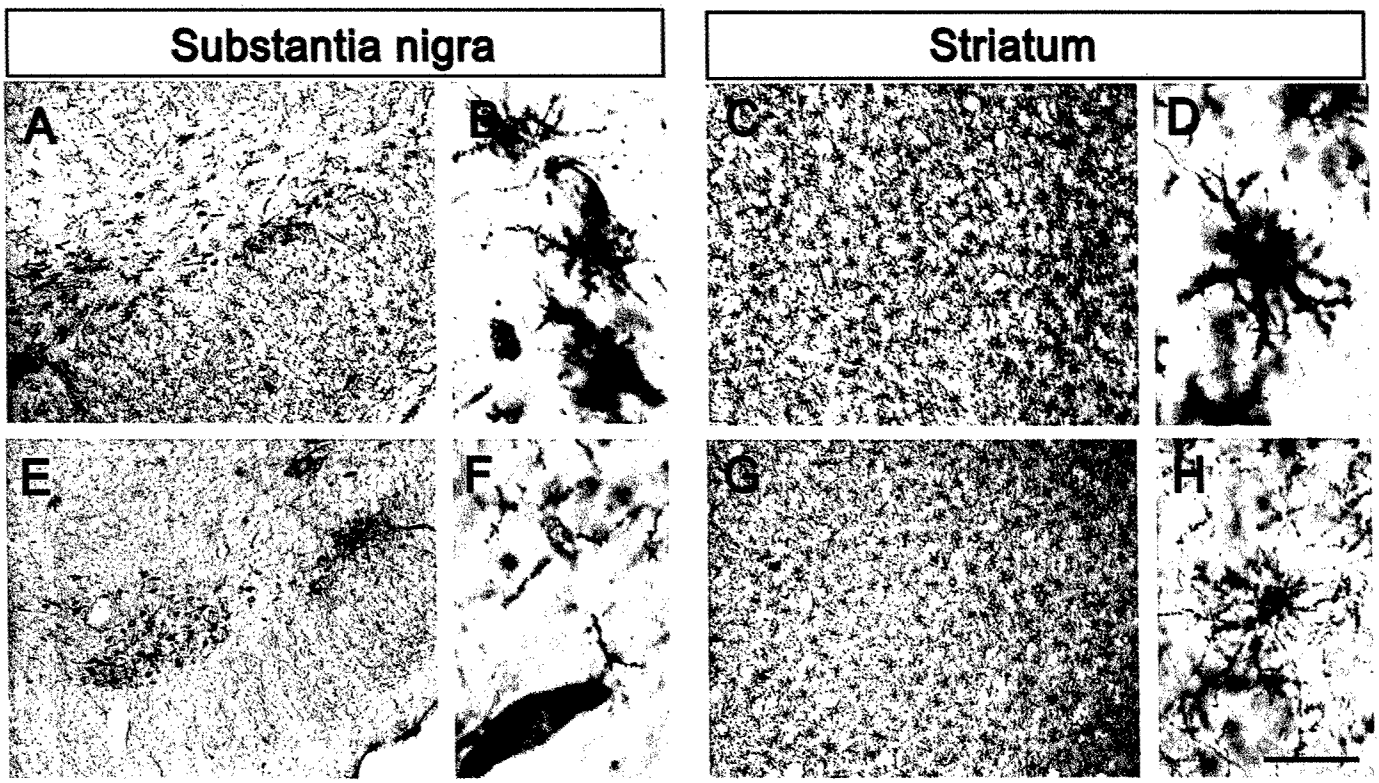

Figure 4. Minocycline prevents MPTP-induced microglia reaction. Microglia cells (*brown*) and TH-positive neurons (*gray blue*) are seen in both SNpc and striatum of all mice. One day after the last MPTP injection, numerous activated microglia (larger cell body, poorly ramified short and thick processes) are seen in SNpc (*A*, *B*) and striatum (*C*, *D*). Mice injected with both MPTP and minocycline show minimal microglial activation in SNpc (*E*) and striatum (*G*); here, microglial cell bodies are small and processes are thin and ramified (*F*, *H*). Scale bar: *A*, *C*, *E*, *G*, 1 mm; *B*, *D*, *F*, *H*, 100 μm.

produced significantly smaller increases in nitrotyrosine levels in ventral midbrains of minocycline (45 mg/kg twice daily)-treated mice than in their non-minocycline-treated counterparts (Table 1). This confirms that minocycline not only attenuates the morphological but also the biochemical impacts of MPTP neurotoxicity.

MPTP metabolism is unaffected by minocycline

The main determining factors of MPTP neurotoxic potency are its conversion in the brain to MPP^+ followed by MPP^+ entry into dopaminergic neurons and its subsequent blockade of mitochondrial respiration (Przedborski et al., 2000). To ascertain that resistance to the neurotoxic effects of MPTP provided by minocycline was not attributable to alterations in any of these three key MPTP neurotoxic steps, we measured striatal levels of MPP^+ 90 min after injection of 18 mg/kg MPTP, striatal uptake of [^{3}H]MPP^+ into synaptosomes, and striatal MPP^+-induced lactate production, a reliable marker of mitochondrial inhibition (Kindt et al., 1987) (Table 2). These investigations showed that striatal levels of MPP^+ did not differ between MPTP-injected mice that either received or did not receive minocycline (45 mg/kg) 30 min after MPTP administration. In addition, minocycline up to 333 μM (maximal solubilizing concentration) did not affect striatal uptake of [^{3}H]MPP^+ or MPP^+-induced lactate production (Table 2).

Minocycline inhibits MPTP-induced microglial activation

To determine whether neuroprotection by minocycline is associated with inhibition of MPTP-induced glial response, we examined the expression of MAC-1, a specific marker for microglia, and GFAP, a specific marker for astrocytes. As shown in Figure 3*B*, MAC-1 mRNA contents ($F_{(3,23)} = 4.252$; $p < 0.05$), but not GFAP mRNA contents ($F_{(3,18)} = 2.843$; $p > 0.05$), varied significantly among the various group of mice. In saline-injected mice, ventral midbrain expression of MAC-1 and GFAP mRNA was minimal (Fig. 3*A*,*B*). In these animals, only a few faintly immunoreactive resting microglia and astrocytes were observed in SNpc and striatum by immunostaining (data no shown). In MPTP-injected mice (18 mg/kg for four injections) without treatment with minocycline, ventral midbrain expression of MAC-1 mRNA was significantly higher, whereas expression of GFAP mRNA, although also higher, was not significantly increased compared with saline controls (Fig. 3). Morphologically, numerous robustly immunoreactive MAC-1-positive activated microglia were observed 24 hr after the last injection of the toxin (Fig. 4*A–D*). Although GFAP immunostaining appeared somewhat increased at 24 hr after the last MPTP injection (Fig. 5*A*,*B*), the strongest GFAP reaction was noted 7 d after the last injection of MPTP (Fig. 5*C*,*D*). Conversely, in MPTP-injected mice treated with minocycline (45 mg/kg twice daily), ventral midbrain MAC-1 mRNA contents (Fig. 3) and SNpc and striatal immunostaining were similar to those seen in saline-injected mice (Fig. 4*E–H*). In contrast, in MPTP-injected minocycline-treated mice, ventral midbrain GFAP mRNA content (Fig. 3) and SNpc immunostaining (Fig. 5*E*,*F*) were almost as high and as intense as in MPTP-only mice. Staining with Isolectin B-4 (Sigma), another marker for microglia, gave results similar to that of MAC-1 (data not shown).

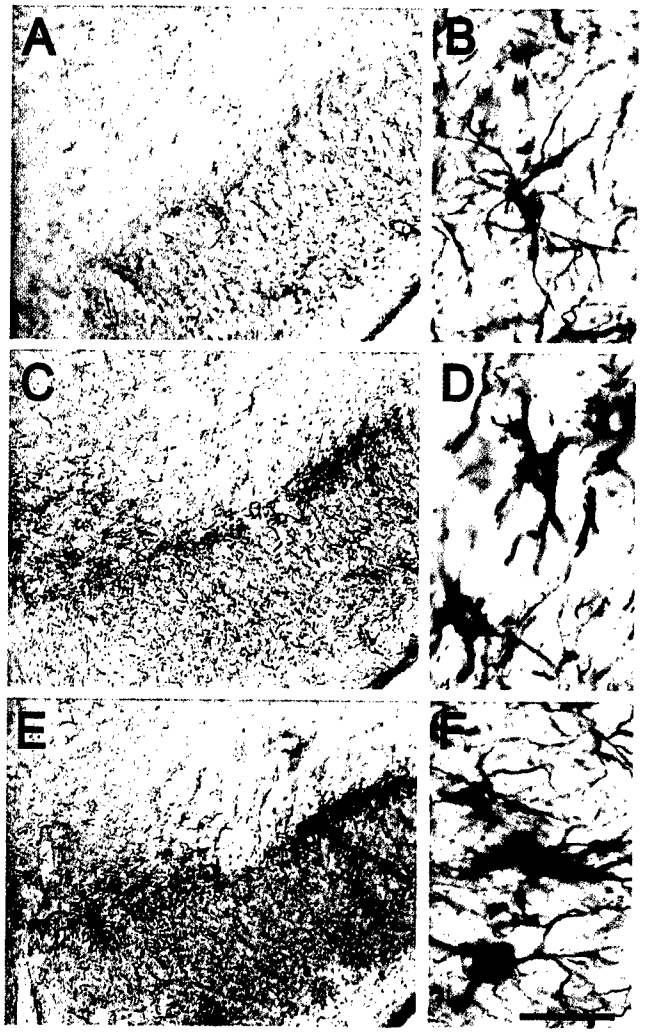

Figure 5. Minocycline does not affect MPTP-induced astrocytic reaction. One day after the last injection of MPTP, there is a mild astrocytic response (*A*, *B*), but 7 d after the last injection of MPTP, it becomes conspicuous (*C*, *D*). Minocycline does not affect the astrocytic response (*E*, *F*) 7 d after MPTP administration. Scale bar: *A*, *C*, *E*, 1 mm; *B*, *D*, *F*, 100 μm.

Minocycline prevents the production of microglial-derived deleterious mediators

Given the effect of minocycline on MPTP-induced microglial activation, we assessed whether the production of known microglial noxious mediators such as IL-1β, ROS, and NO will also be inhibited by minocycline (Fig. 6). The levels of ventral midbrain IL-1β differed significantly among the four group of mice ($F_{(3,21)} = 7.946$; $p < 0.001$) (Fig. 6*A*). Ventral midbrain levels of the proinflammatory cytokine IL-1β in MPTP-injected mice (18 mg/kg for four injections) were significantly increased (Fig. 6*A*). However, MPTP produced significantly smaller increases in IL-1β levels in ventral midbrain of MPTP mice treated with minocycline (45 mg/kg twice daily) (Fig. 6*A*). iNOS activity ($F_{(3,24)} = 9.055$; $p < 0.001$) and the ratio of membrane/total $p67^{phox}$ ($F_{(3,23)} = 4.336$; $p < 0.05$) also varied significantly among the various groups. iNOS and NADPH–oxidase, two prominent enzymes of activated microglia that produce NO and ROS, respectively, exhibited induction patterns similar to those described for IL-1β in that ventral midbrain iNOS activity was increased by 200% (Fig. 6*B*) and NADPH–oxidase activation, evidenced by the translocation of its subunit $p67^{phox}$ from the cytosol to the plasma membrane, was increased by 80% 24 hr after the last injection of MPTP (Fig. 6*C*,*D*). MPTP-induced iNOS activity and NADPH–oxidase were both abolished by minocycline administration (Fig. 6*B–D*).

Minocycline confers resistance to MPTP beyond iNOS ablation

Previously, it has been demonstrated that iNOS ablation attenuates MPTP neurotoxicity (Liberatore et al., 1999; Dehmer et al., 2000). Thus, to demonstrate whether minocycline-mediated blockade of microglial activation protects solely because it inhibits iNOS induction, we compared the effect of MPTP (16 mg/kg for four injections) on the network of striatal dopaminergic nerve fibers between mutant iNOS-deficient mice that received or did not receive minocycline (45 mg/kg twice daily). As shown in Figure 7, MPTP administration reduced by >80% the striatal density of TH-positive fibers both in wild-type and $iNOS^{-/-}$ mice; this is consistent with our previous data that ablation of iNOS protects against MPTP-induced SNpc dopaminergic neuronal loss but not against MPTP-induced striatal dopaminergic fiber destruction (Liberatore et al., 1999). In contrast, striatal TH-positive fiber densities were more than twofold higher in MPTP-treated wild-type and $iNOS^{-/-}$ mice that received minocycline compared with those that did not receive minocycline (Fig. 7). However, there was no difference in the magnitude of the minocycline beneficial effect between MPTP-treated $iNOS^{-/-}$ mice and their MPTP-treated wild-type counterparts (Fig. 7).

DISCUSSION

The main finding of this study is that inhibition of microglial activation by minocycline protects the nigrostriatal dopaminergic pathway against the noxious effects of the parkinsonian toxin MPTP. In mice that received minocycline, MPTP caused significantly less neuronal death in the SNpc, as evidenced by the greater number of TH-positive neurons, compared with those that received MPTP only (Fig. 1). Although less prominent, a similar observation was made for striatal dopaminergic nerve terminals (Fig. 2). The magnitude of resistance to MPTP in mice appears to result from a balance between the dose of minocycline and the dose of MPTP (Fig. 1), with the greatest neuroprotection observed in mice that received >11.25 mg/kg minocycline twice daily and MPTP at 16 mg/kg four times in 1 d and the least neuroprotection in mice that received the regimen of minocycline at 6.1 mg/kg twice daily and MPTP at 18 mg/kg four times in 1 d. In our study, minocycline was given twice daily beginning on the day of MPTP administration and continuing through 4 d thereafter because of its long half-life (>12 hr) and because we showed that, with this MPTP regimen, nigrostriatal degeneration occurs during the first 4 d after the last injection of MPTP (Jackson-Lewis et al., 1995). Therefore, we cannot exclude that greater protection could have been achieved if minocycline had been administered more frequently or for a longer period of time. Also, because we focused our assessment of nigrostriatal neurodegeneration at 7 d after MPTP administration, we cannot exclude with certainty that minocycline had delayed rather than prevented neuronal death. However, in light of what we know about how minocycline presumably mitigates cellular damage in a variety of experimental models (Tikka and Koistinaho, 2001; Tikka et al., 2001a), the aforementioned possibility appears unlikely. In addition, we did not pretreat mice with minocycline

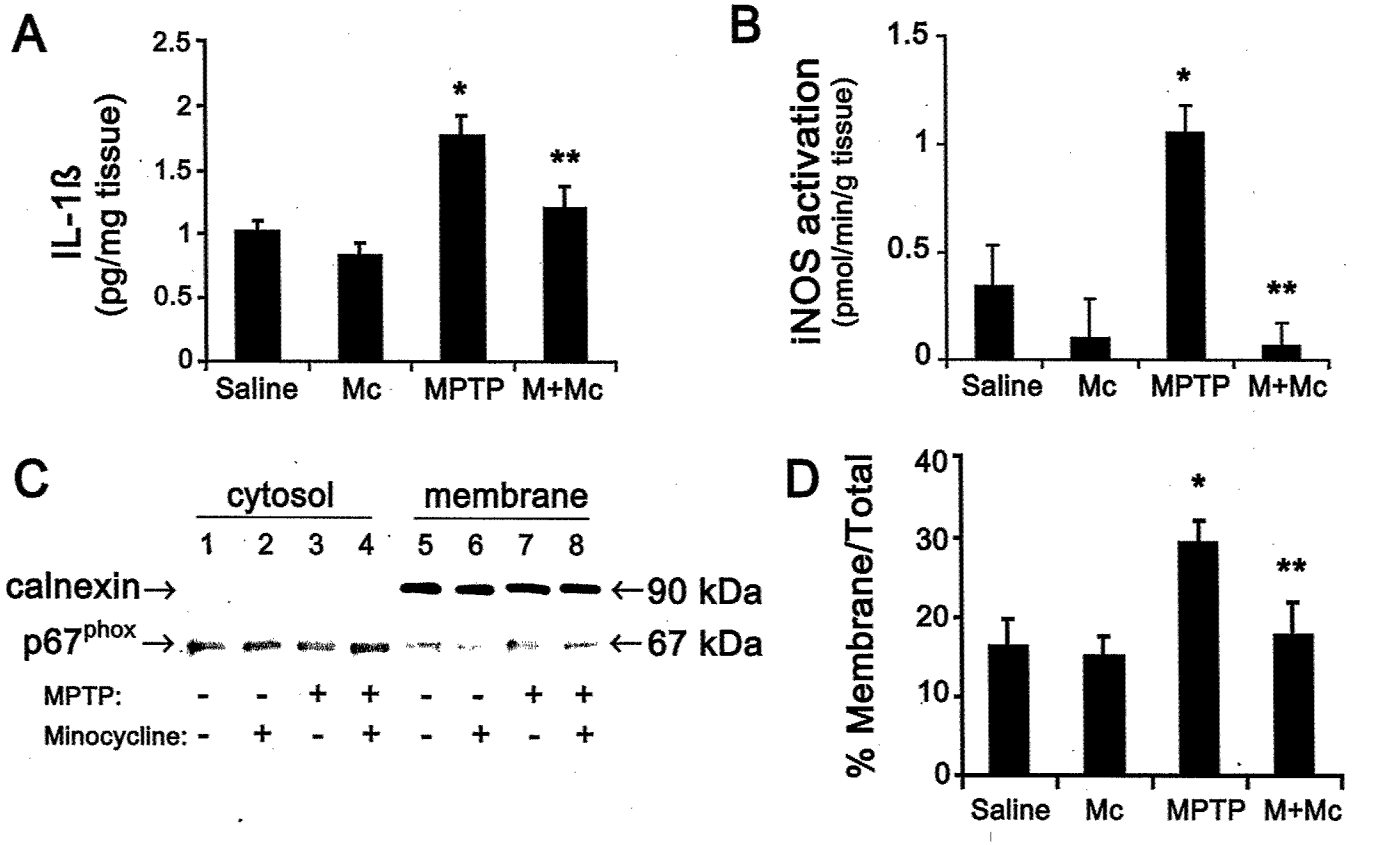

Figure 6. Effects of MPTP and minocycline on microglial-derived deleterious factors IL-1β (*A*), iNOS (*B*), and NADPH–oxidase (*C*, *D*). MPTP (18 mg/kg for 4 injections) increases ventral midbrain mature IL-1β formation, iNOS catalytic activity, and NADPH–oxidase activation, as evidenced by the translocation of its subunit $p67^{phox}$ from the cytosol to the plasma membrane, 1 d after the last injection of MPTP. Minocycline (45 mg/kg twice daily) attenuates MPTP-related effects on mature IL-1β, iNOS, and NADPH–oxidase. *Saline*, Saline-treated; *Mc*, minocycline-treated; *M*, MPTP-treated; *M+Mc*, MPTP plus minocycline-treated. *$p < 0.05$, more than saline-injected or minocycline-injected control mice. **$p < 0.05$, less than MPTP-injected mice but not different from both control groups. Values are means ± SEM (n = 5–8 mice per group).

because we found that administration of minocycline before MPTP injection reduces striatal MPP^+ levels by 20% (Table 2), which could complicate the interpretation of minocycline neuroprotection. Indeed, it is established that striatal contents of MPP^+ correlate linearly with magnitudes of MPTP toxicity (Giovanni et al., 1991). Thus, to avoid this potential confounding factor in our study, all mice were injected first with MPTP and then with minocycline, which we found not to affect striatal MPP^+ levels (Table 2). Along this line, it is also worth mentioning that minocycline, as used here, not only failed to alter MPP^+ levels but also failed to interfere with other key aspects of MPTP metabolism (Przedborski et al., 2000), such as entry of MPP^+ into dopaminergic neurons and inhibition of mitochondrial respiration at concentrations as high as 333 μM (Table 2).

Nitrotyrosine is a fingerprint of NO-derived modification of protein and has been documented as one of the main markers of oxidative damage mediated by MPTP (Schulz et al., 1995; Ara et al., 1998; Liberatore et al., 1999; Pennathur et al., 1999; Przedborski et al., 2001a). Consistent with our previous studies (Liberatore et al., 1999; Pennathur et al., 1999), nitrotyrosine levels increased substantially in brain regions affected by MPTP, such as ventral midbrain, but not in brain regions unaffected by MPTP, such as cerebellum (Table 1). As with the loss of SNpc neurons and striatal fibers, minocycline dramatically attenuated ventral midbrain increases in nitrotyrosine levels (Table 1). Collectively, our data demonstrate that minocycline protects against morphological as well as biochemical abnormalities that arise

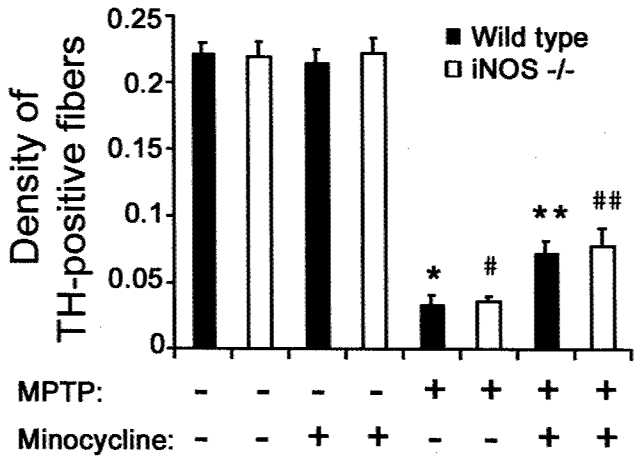

Figure 7. Minocycline attenuates MPTP-induced striatal damage by inhibiting microglia but not just by inhibiting iNOS. The optical density of striatal TH-positive fibers varied significantly among the various groups ($F_{(7,47)} = 83.576$; $p < 0.001$). *Minocycline*, Mice injected with minocycline 45 mg/kg twice daily. MPTP, Mice injected with MPTP (4 injections of 16 mg/kg). *$p < 0.05$, fewer than saline-injected or minocycline-injected control mice. #$p < 0.05$, fewer than control mice but no different than wild-type mice injected with MPTP. **$p < 0.05$, fewer than control but more than MPTP-injected mice. # #$p < 0.05$, more than MPTP-injected mice but no different from wild-type mice injected with both MPTP and minocycline.

from MPTP insult. That said, we now need to consider the nature of the mechanism underlying the beneficial effects of minocycline on MPTP neurotoxicity.

Previously, we demonstrated that, aside from a dramatic loss of dopaminergic neurons, gliosis is a striking neuropathological feature in the SNpc and the striatum in the MPTP mouse model as in PD (Liberatore et al., 1999). However, activated microglia appear in the SNpc earlier than reactive astrocytes (Liberatore et al., 1999) and at a time when only minimal neuronal death occurs (Jackson-Lewis et al., 1995). This supports the contention that the microglial response to MPTP arises early enough in the neurodegenerative process to contribute to the demise of SNpc dopaminergic neurons. Consistent with this is the demonstration that direct injection of the known microglial activator lipopolysaccharide into the rat SNpc causes a strong microglial response associated with significant dopaminergic neuronal death (Castano et al., 1998; Herrera et al., 2000; Kim et al., 2000). Given these data, the key to the minocycline neuroprotective effect in the MPTP mouse model may lie in the second main finding of our study, which is that minocycline prevented MPTP-induced microglial response in both the SNpc and the striatum (Figs. 3, 4). In contrast, minocycline did not alter MPTP-related astrocytic response (Fig. 5). These results suggest that minocycline acts on microglia specifically and not on all components of gliosis. Our data also support the view that reduction of MPTP-related microglial response seen after minocycline administration is not secondary to the attenuation of neuronal loss but rather the reverse. This interpretation does not rule out, however, that at least some of the neuroprotection of minocycline against MPTP is attributable to a direct action on neurons as suggested previously (Tikka et al., 2001b).

Inhibition of microglial activation using minocycline has also been demonstrated *in vitro* (Tikka et al., 2001b) and in other experimental models of acute and chronic brain insults (Yrjanheikki et al., 1998, 1999; Tikka and Koistinaho, 2001; Tikka et al., 2001a) and results, presumably, from the blockade of p38 mitogen-activated protein kinase (Tikka et al., 2001a). It is believed that activated microglia exerts cytotoxic effects in the brain through two very different and yet complementary processes (Banati et al., 1993). First, they can act as phagocytes, which involve direct cell-to-cell contact. Second, they are capable of releasing a large variety of potentially noxious substances (Banati et al., 1993). Consistent with the notion that minocycline inhibits the ability of microglia to respond to injury, we show that minocycline not only prevents the microglial morphological response to MPTP but also the microglial production of cytotoxic mediators such as IL-1β and the induction of critical ROS- and NO-producing enzymes such as NADPH–oxidase and iNOS (Fig. 6). Although we did not test this, it is quite relevant to mention that minocycline may also prevent the induction of cyclooxygenase-2, a key enzyme in the production of potent proinflammatory prostanoids, either directly or indirectly via the blockade of IL-1β formation (Yrjanheikki et al., 1999). Little is known about the actual role of IL-1β in either MPTP or PD neurodegenerative process, except that IL-1β immunoreactivity is found in glial cells from postmortem PD SNpc samples (Hunot et al., 1999) and that blockade of interleukin converting enzyme, the known activator of IL-1β, attenuates MPTP-induced neurodegeneration in mice (Klevenyi et al., 1999). As for ROS, oxidative stress is a prominent pathogenic hypothesis in both MPTP and PD (Przedborski and Jackson-Lewis, 2000). However, many of the microglial-derived ROS, such as superoxide, cannot readily transverse cellular membranes (Halliwell and Gutteridge, 1991), making it unlikely that these extracellular reactive species gain access to dopaminergic neurons and trigger intraneuronal toxic events. Alternatively, superoxide can react with NO in the extracellular space to form the highly reactive tissue-damaging species peroxynitrite, which can cross the cell membrane and injure neurons. Therefore, microglial-derived superoxide, by contributing to peroxynitrite formation, may be significant in this model. As for NO in both MPTP and PD, the pivotal pathogenic role for microglial-derived NO is supported by the demonstration that ablation of iNOS attenuates SNpc dopaminergic neuronal death (Liberatore et al., 1999; Dehmer et al., 2000) and the production of ventral midbrain nitrotyrosine after MPTP administration (Liberatore et al., 1999). In this context, it is worth mentioning that minocycline, which protects in global brain ischemia (Yrjanheikki et al., 1998) and in a mouse model of Huntington's disease (Chen et al., 2000), appears to do so by abating iNOS expression and activity. Remarkably, iNOS ablation does protect SNpc neurons from MPTP toxicity but does not protect striatal nerve terminals and does not prevent microglial activation (Liberatore et al., 1999). This is in striking contrast to the effect of minocycline treatment, which protects both dopaminergic cell bodies and nerve fibers and inhibits the entire microglial response. This strongly suggests that microglial-associated deleterious factors other than iNOS are involved in the demise of the nigrostriatal pathway in the MPTP mouse model of PD and possibly in PD itself. Consistent with this interpretation are our data in iNOS$^{-/-}$ mice (Fig. 7), which show that minocycline protects striatal dopaminergic fibers regardless of the presence or absence of iNOS expression. Therefore, our study provides strong support to the idea that activated microglia are important contributors to the overall demise of SNpc dopaminergic neurons in the MPTP mouse model of PD and, possibility, in PD itself. It also suggests that therapeutic interventions aimed at preventing the loss of striatal dopaminergic fibers, which is essential to maintaining dopaminergic neurotransmission, must target microglial-derived factors other than iNOS.

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The Last Decade in Parkinson's Disease Research

Basic Sciences

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Parkinson's disease (PD) is a common neurodegenerative disorder whose cardinal features include tremor, slowness of movement, stiffness, and poor balance (1). Most, if not all, of these disabling symptoms are secondary to a profound reduction in striatal dopamine content, caused by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) and of their projecting nerve fibers in the striatum (2,3). Although several approved drugs do alleviate PD symptoms, their chronic use is often associated with debilitating side effects (4), and none seems to dampen the progression of the disease. Moreover, the development of effective preventive or protective therapies is impeded by our limited knowledge of the cause (i.e., etiology) and mechanisms (i.e., pathogenesis) by which dopaminergic neurons die in PD.

Although neither the etiology nor the pathogenesis of PD has yet been elucidated, this last decade has witnessed an explosion of invaluable research, which unquestionably has provided critical insights into our current understanding of this illness. Accordingly, in this chapter, we give an overview of what we believe the key findings of the past 10 years in PD are. We also try to place each of these findings within the context of what appears to be the best to date, the direction in which the field of PD research seems to be evolving. One caveat of our approach resides in the fact that the goal of this chapter is to provide a "flavor" for the field of PD research rather than a comprehensive review. Therefore, the reader must be aware that only selected aspects of the research performed in PD are reviewed and discussed. Along this line, the reader should also know that this chapter does not, except incidentally, review the large core of research dealing with "symptomatic therapies," whether the approach is pharmacological or surgical, which are discussed elsewhere in this book.

ETIOLOGY OF PD

If the goal is to prevent PD and to diagnose it before any actual neurodegeneration occurs, then we must unravel the etiology of this disorder. For many years, the two main hypotheses for the etiology of PD that have prevailed have been the toxic and the genetic hypotheses. As we will see, there is supportive evidence for both hypotheses, and neither one is exclusive of the other.

Toxic Hypothesis

According to this hypothesis, it is proposed that a deleterious compound may be present in our environment, even in low amounts, and

that over time it may accumulate in our organism and ultimately reach a threshold level that will cause it to unleash its damaging properties against the dopaminergic system. A significant support for an "exogenous or environmental toxin" has been provided by the discovery that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) can cause a parkinsonian syndrome in humans almost identical to PD (5). Also relevant is the fact that 1-methyl-4-phenylpyridimium (MPP^+), MPTP's active metabolite, has been extensively used as an herbicide in many countries around the world, as has paraquat, which has a striking structural similarity to MPP^+. Furthermore, a rural environment has often been found to be associated with an elevated risk of PD (6). In keeping with this, factors such as herbicides, pesticides, and well water have all been incriminated in the occurrence of PD (6). To date, it remains, however, that despite all of these supportive observations, no actual compound, related to the MPTP family or not, has been unequivocally linked to the development of PD. Alternatively, it has also been proposed that perhaps the putative parkinsonian toxin is not exogenous but rather is produced by our own metabolism, giving rise to the "endogenous toxin" hypothesis of PD (7). According to this model, a noxious compound would be produced in response to either a defective or a variant metabolic pathway. In keeping with this view is the suggestion that patients harboring specific polymorphisms in the gene encoding for the cytochrome P450 may be at greater risk of developing young-onset PD (8). Furthermore, several isoquinoline derivatives, which can kill dopaminergic neurons, have been recovered from the brains of PD patients and thus are regarded by several experts as potential endogenous parkinsonian neurotoxins (9). Among these, tetrahydroisoquinoline (TIQ), 1-benzyl-TIQ, and *(R)*-1,2-dimethyl-5,6-dihydroxy-TIQ have the most potent neurotoxic effects (9).

Genetic Hypothesis

During the last decade, there has been a clear waning and waxing of enthusiasm for the role of genetics in the etiology of PD. However, during the past few years there has been an upsurge of interest in the genetics of PD, triggered by several breakthroughs obtained in familial forms of parkinsonism. For instance, point mutations in the α-synuclein gene, located on chromosome 4, have been found to cause an autosomal dominant parkinsonian syndrome (10,11). The two missense mutations identified thus far result in a single amino acid substitution in α-synuclein protein, that is, an alanine being replaced by a hydrophobic residue threonine at position 53 and by proline at position 30. Since the discovery of these mutations, data have been accumulated suggesting that both mutations may alter α-synuclein's normal intracellular distribution, enhance α-synuclein's propensity to interact with other intracellular proteins, and increase α-synuclein's disposition to aggregate and consequently to form intraneuronal inclusions (12–16). However, thus far, efforts to identify α-synuclein mutations in sporadic PD have failed (17–19). On the other hand, in sporadic PD, α-synuclein has been demonstrated to be a major component of the intraneuronal inclusions Lewy bodies, which are a pathologic hallmark of the disease (20,21). In addition, we have recently demonstrated that α-synuclein is up-regulated in dopaminergic neurons of the SNc after MPTP administration to mice (22). However, the normal function of this protein and its implications for the pathogenesis of PD remain to be determined.

The gene for an autosomal recessive form of juvenile parkinsonism has also recently been identified and encoded for a protein called parkin (23). More recently, a susceptibility locus for PD has been mapped to chromosome 2p13 (24), and a point mutation in the gene encoding for a key enzyme of the ubiquitin pathway has been identified in a family with parkinsonism (25). In light of all of these discoveries, we can conclude that there is increasing evidence that genetic factors might play a role in PD. However, these studies also show quite clearly that only a small number of the multigenerational fam

d in the cited investigations have con- pathology of PD, and most of the af- family members exhibit atypical fea- such as an onset at younger age, rapid and often dementia. Moreover, the of inheritance of the parkinsonism these families is highly variable, rang- rom autosomal dominant to autosomal sive and even the possibility of being rnally transmitted (26), raising the poten- y of a genetic defect within the mito- ndrial genome. Because, to date, none of identified mutations found in familial PD been identified in sporadic PD, it is likely t these genetic alterations may account, at ost, for a very small fraction of PD patients. evertheless, these findings remain ex- emely exciting, as they raise the prospect that by elucidating the actual mechanisms by which these genetic defects produce the demise of dopaminergic neurons in familial forms of PD, they may well shed light into the etiology and pathogenesis of sporadic PD.

In our opinion, one of the most damaging arguments against a pivotal genetic component in the etiology of PD is provided by the lack of significant concordance in monozygotic twins with classical PD (27). It remains plausible that genetics may play a critical role, not as a unique etiologic factor but rather within a multifactorial cascade such as through an interaction between genetic and toxic mechanisms. This view is supported by the demonstration that individuals carrying a specific mitochondrial mutation will develop deafness only on exposure to aminoglucoside (28). Conversely, it is also relevant to point out that, among the cohort of individuals who were intoxicated with MPTP, only a fraction developed parkinsonism (Dr. J. W. Langston, *personal communication*). These two examples emphasize the potential importance of the interaction between genetic and toxic factors.

PATHOGENESIS OF PD

In spite of the above-described efforts in identifying the etiology of PD, affected patients to date are diagnosed only when the symptoms of the disease have already appeared. Therefore, it is important to identify the mechanisms involved in the cellular death in order to halt or slow down the progression of the disease once it is already established.

In an attempt to unravel the mechanisms implicated in the neurodegenerative process in PD, a large number of presumed pathogenic factors have been put forward, including dopamine metabolism, mitochondrial dysfunction, free radicals, cell death by apoptosis, excitotoxicity, defect in trophic factors, and many others. Some of these are discussed below.

Dopamine Metabolism

Dopamine is the neurotransmitter of the SNc neurons controlling normal motor function. It seems, however, that dopamine is not essential for the normal development of the SNc system, as observed in mice lacking tyrosine hydroxylase (TH), the enzyme catalyzing the first and rate-limiting step of catecholamine biosynthesis. It is worthy to note that although these mutant mice have almost no dopamine production, they show a normal cytoarchitecture of the SNc dopaminergic system (29,30) as evidenced by immunostaining for DOPA decarboxylase, another enzyme in this synthetic pathway. Interestingly, between 2% and 22% of wild-type catecholamine concentrations are found in the brains of these mutant (31) mice, likely as the result of alternative synthetic pathways such as that involving tyrosinase, another enzyme that converts tyrosine to L-DOPA but that does so during melanin synthesis.

In the mature brain, although it is clear from PD that dopamine is essential for motor control, it has been frequently suggested that dopamine at the same time may exert deleterious effects that may participate in the progression of the disease. Evidence in support of this view is still lacking *in vivo* in that there is no definitive demonstration that individuals who erroneously receive high doses of dopamine precursor, L-DOPA, for a pro-

longed period fare worse because of the drug. Similarly, the toxic effect of dopamine/L-DOPA has not been observed in studies using animals with intact nigrostriatal pathway. More confusing is the situation in rats with moderate SNc damage produced by the neurotoxin 6-hydroxydopamine, which generated contradictory results (32,33). The issue of dopamine-mediated toxicity is more compelling when one looks at the large core of *in vitro* studies dealing with this question. For instance, it has been shown that 200 μM of L-DOPA can cause a 50% reduction in the number of dopaminergic neurons in postnatal midbrain cultures (34). This toxic effect seems to be mediated by the production of free radicals because it is prevented by the overexpression of copper/zinc superoxide dismutase (SOD1) (34), a key free radical–scavenging enzyme.

Another intriguing aspect related to the dopamine metabolism that has not yet been solved concerns the link between the vulnerability of SNc dopaminergic neurons and the prominent content in black pigmentation called neuromelanin in these neurons (35). It has been reported that (a) the dopamine-containing cell groups of the normal human midbrain differ markedly from each other in the percentage of neuromelanin-pigmented neurons they contain; (b) the estimated cell loss in these cell groups in PD is directly correlated with the percentage of neuromelanin-pigmented neurons normally present in them; and (c) within each cell group in PD brains, there is greater relative sparing of nonpigmented than of neuromelanin-pigmented neurons (36). These results suggest a selective vulnerability of the neuromelanin-pigmented subpopulation of mesencephalic dopaminergic neurons in PD. To date, however, the role of neuromelanin within dopaminergic cells and its origin are not known. A new insight in this field comes from the observation that, in postnatal midbrain cultures exposed to low doses of L-DOPA, dopaminergic neurons accumulate a black pigment with the same characteristics as neuromelanin (36a). This finding indicates that the formation of neuromelanin is clearly related to the presence of L-DOPA/dopamine, and thus, this *in vitro* cellular system may represent a new tool with which to study the actual role played by neuromelanin in the neurodegenerative process. Another unresolved issue inherent to dopaminergic neuron degeneration in PD is the potential contribution of Lewy bodies in the death of these neurons. Along this line, Dr. Sulzer's group has also found that incubation of monoaminergic clonal PC-12 cells with L-DOPA induces ubiquitinated intracellular inclusions reminiscent of Lewy bodies. This exciting finding may enable us to identify the factors involved in the formation of the Lewy bodies as well as to determine their actual role in the neurodegenerative process.

Dopamine metabolism by monoamine oxidase or by autooxidation leads to the formation of hydrogen peroxide, superoxide radicals, and several reactive quinones and semiquinones that could contribute to the heightened state of oxidative stress in PD (37). Neuromelanin within dopaminergic neurons can bind ferric iron and reduce it to its reactive ferrous form (35). Taken together, these results show that the SNc, because of its dopamine and neuromelanin content, is a designated target for oxidative attack. This view leads us now to discuss the important questions of oxidative stress and of mitochondrial dysfunction in PD (37).

Oxidative Stress and Mitochondrial Dysfunction

Several lines of evidence suggest that the SNc in PD is the site of an oxidative stress (37). As mentioned above, several powerful oxidants are produced in the course of normal metabolism, including hydrogen peroxide, superoxide, peroxyl and hydroxyl, and even nitric oxide (NO). These molecules may cause cellular damage by reacting with nucleic acids, proteins, lipids, and other molecules. Indeed, in the SNc of parkinsonian patients there is evidence of increased malondialdehyde and hydroperoxidase, which suggests lipid peroxidation, increased carbonyl proteins suggesting oxidized proteins, increased

oxy-2-deoxyguanosine suggesting DNA e, elevation of iron levels, increase in γ- nyl transpeptidase activity, and dimin- reduced glutathione. The possibility that tive stress participates in the pathogene- PD offers therapeutic strategies based in use of antioxidant agents in order to pro- neuroprotection. These may include free ical scavengers, glutathione-enhancing ents, iron chelators, and drugs that interfere ith the oxidative metabolism of dopamine. o date, clinical trials have been performed ith vitamin E and deprenyl but have failed to how definite neuroprotection.

One main source of reactive oxygen species is the mitochondria. It is thus relevant to mention that a reduction in the activity of the complex I (NADH-ubiquinone oxidoreductase) of the mitochondrial electron transport chain in PD brains has been reported (38). This defect could subject cells to oxidative attack as well as energy failure. Furthermore, it seems that the mitochondrial defect found in PD is generalized and not confined to the brain, as reduced complex I activity has been reported in platelets from PD patients. In addition, hybrid cells, in which mitochondrial DNA has been destroyed and repopulated with mitochondrial DNA from the platelets of PD patients, reproduce the defect in complex I activity (39). The latter finding suggests that the observed complex I deficit originates from an alteration in the mitochondrial rather than the nuclear genome.

It is of importance to indicate that although mitochondrial dysfunction and oxidative metabolism may well be critical components in the cascade of deleterious events leading to the death of SNc dopaminergic neurons, surprisingly none of the data available to date did address the question as to whether these abnormalities represent a primary or secondary events. Indeed, all of these data are merely circumstantial and correlative observations reported in autopsied brains in which most of the dopaminergic cells have already been destroyed, and thus the mechanistic value of autopsy findings must be taken with a great deal of caution.

Programmed Cell Death

In recent years, there has been growing interest in the manner in which neuronal cells degenerate. In this context, the concept that programmed cell death (PCD) may play a role in the pathogenesis of neurodegenerative disorders has emerged as an important hypothesis. PCD represents an active form of cell death in which intrinsic cellular genetic programs are activated, leading to cellular "suicide." This form of death must be distinguished from the presumed passive cellular death resulting from a noxious effect or harsh environmental factors. PCD is also referred to as apoptosis because apoptosis is probably the most common morphologic type of PCD. However, it is important to mention that apoptosis refers to a specific set of morphologic features and is not the sole and unique morphologically defined form of death encountered in PCD (40). For instance, apoptosis is defined by the association of cell body and nucleus shrinkage, chromatin clump formation, DNA fragmentation, and condensation of cytosol and nucleosol, often with preservation of organelles and phenotypic markers. The question of whether apoptosis occurs in neurodegenerative disorders should not be regarded as an esoteric academic problem but rather as a line of research that can shed light into the pathogenesis of PD as well as open new therapeutic avenues.

It has been reported that apoptosis occurs in the substantia nigra during normal development in rodents (41,42). It has also been demonstrated that this phenomenon is time dependent, paralleling the time-course of synaptogenesis, is modulated by factors derived from the target (and/or postsynaptic neurons), and occurs in dopaminergic neurons *per se* as evidenced by TH immunostaining (41–44). Occurrence of SNc PCD has also been examined in mature brains by studying experimental models of PD. Along this line, it has been reported that intrastriatal injection of 6-hydroxydopamine (6-OHDA) in developing animals results in the induction of apoptosis in SNc dopaminergic neurons (45). This effect

seems to be explained by the destruction of dopaminergic terminals by 6-OHDA, thus interfering with target support, rather than a direct action of the toxin-inducing apoptosis. The ability of intrastriatal 6-OHDA to induce apoptotic death is developmentally dependent, with a major induction of death during the first two postnatal weeks but only a minor effect at later postnatal times. Furthermore, at later postnatal days, 6-OHDA-induced cell death presented two different morphologies, apoptotic and nonapoptotic. This suggests either that the toxin induces cell death by two different mechanisms or that the same fundamental mechanism induces an apoptotic morphology in less mature animals and a nonapoptotic morphology in more mature animals. Studies with MPTP in mice have reported mixed results. We initially reported that acute administration of MPTP, in which the drug was given in four separate doses administered every 2 hours, resulted in a nonapoptotic cell death in the SNc (46). More recently, Tatton and Kish have reported (47) in a chronic model of MPTP administration (30 mg/kg per day for five consecutive days) the occurrence of apoptosis in phenotypically defined dopaminergic neurons. Therefore, PCD plays a role in the MPTP mouse model of PD, depending on the administration schedule of the neurotoxin. Finally, in PD, the situation is more complex in that there is mixed evidence concerning whether apoptotic morphology can be identified in the postmortem PD brains of patients (48). It is important to note that apoptotic cell death seems to take place within a short period of time, making its identification difficult in a chronic degenerative disease, and that the quality of the autopsied material might not allow high-quality morphologic studies to be performed.

The MPTP Mouse Model of PD

The fact that MPTP causes a parkinsonian syndrome was discovered in 1982 when a group of drug addicts in California exhibited a severe and irreversible akinetic rigid syndrome analogous to PD (49). Subsequently, it was found that this syndrome was induced by the self-administration of a synthetic heroin analog whose synthesis had been heavily contaminated by a by-product, MPTP (5). Since then, MPTP has been used extensively as a model of PD (5,50,51). From neuropathologic data, MPTP administration causes damage to the dopaminergic pathways identical to that seen in PD (52). Like PD, MPTP causes a greater loss of dopaminergic neurons in the SNc than in the ventral tegmental area (53,54) and a greater degeneration of dopaminergic nerve terminals in the putamen than in the caudate nucleus (55). On the other hand, the eosinophilic intraneuronal inclusions, Lewy bodies, so characteristic of PD, have thus far not convincingly been observed in MPTP-induced parkinsonism (56). However, MPTP has never been recovered from postmortem brain samples or body fluids of PD patients, consistent not with MPTP causing PD but with its being an excellent experimental model of PD. Accordingly, it can be speculated that elucidating the molecular mechanisms of MPTP should lead to important insights into the pathogenesis and treatment of PD.

The metabolism of MPTP is a complex, multistep process (57). After its systemic administration, MPTP, which is highly lypophilic, rapidly crosses the blood–brain barrier and, once in the brain, this protoxin is metabolized to 1-methyl-4-phenyl-2,3-dihydropyridimium ($MPDP^+$) (by the enzyme monoamine oxidase type B) and then to MPP^+. Thereafter, MPP^+ gains access to dopaminergic neurons by binding to plasma membrane dopamine transporter (DAT) (58). The obligatory character of this step in the MPTP neurotoxic process is demonstrated by the fact that blockade of DAT by specific antagonists such as mazindol (59) or ablation of DAT gene in mutant mice (60) completely prevents MPTP-induced toxicity. Conversely, transgenic mice with increased brain DAT expression are more sensitive to MPTP (61).

Inside dopaminergic neurons, MPP^+ can be concentrated by an active process within the mitochondria (62), where it impairs mitochondrial respiration by inhibiting complex

the electron transport chain (63–65). The ibition of complex I impedes the flow of trons along the mitochondrial electron sport chain, leading to a deficit in ATP mation. It appears, however, that complex I tivity must be reduced by at least 70% to use severe ATP depletion (66) and that, in ontrast to the situation *in vitro, in vivo* MPTP auses only a transient 20% reduction in mouse striatal and midbrain ATP levels (67). Another consequence of complex I inhibition by MPP^+ is an increased production of free radicals, especially of superoxide (68–70). The importance of MPP^+-related superoxide production in the dopaminergic toxicity process *in vivo* is demonstrated by the fact that transgenic mice with increased brain activity of SOD1 are significantly more resistant to MPTP (71). However, superoxide is poorly reactive, and it is the general consensus that this radical does not cause serious direct injury (72). Instead, superoxide is believed to exert many or most of its toxic effects through the generation of other reactive species such as hydroxyl radical, whose oxidative properties can ultimately kill cells (72).

Superoxide can also react with NO to produce peroxynitrite, a potent oxidant (73). In light of this and of our previous work on superoxide (71), we (74) and others (75,76) have assessed the role of NO in the MPTP neurotoxic process. These studies show that inhibition of NO synthase (NOS) by 7-nitroindazole (7-NI), a compound that inhibits NOS activity without significant cardiovascular effects in mice (77), attenuates, in a dose-dependent fashion, MPTP-induced dopaminergic toxicity (74,75). The protective effect of the NOS antagonist 7-NI against MPTP-induced dopaminergic damage was subsequently demonstrated in monkeys (76).

Neuronal NOS (nNOS) is the predominant isoform of NOS in the brain (78,79). Both by its abundance and its localization, nNOS appears to be an excellent candidate for producing NO for MPTP; in agreement with this possibility is our demonstration that mutant mice deficient in nNOS are partially protected against MPTP (74). The finding that mice are better protected by the NOS antagonist 7-NI than by the lack of nNOS expression suggests that isoforms other than nNOS may also be involved in MPTP neurotoxic process. Consistent with this view, it should be mentioned that inducible NOS (iNOS), which is not or is only minimally expressed in normal brains (80,81), is dramatically up-regulated after injury including that produced by MPTP (82). Indeed, early in the course of MPTP-induced dopaminergic neuron degeneration, there is an increase in midbrain iNOS activity within the robust glial reactions that occur in the SNc following the administration of this toxin (82). Consistent with the important role of iNOS in the MPTP neurotoxic process is our demonstration that mutant mice deficient in iNOS are more resistant to MPTP (82).

Among the various forms of damage produced by peroxynitrite, the presumed culprit in MPTP-mediated toxicity, is the oxidation of phenolic rings in proteins, and in particular of tyrosine residues (83), to form nitrotyrosine as the most important product (84). Thus, detection and quantification of nitrotyrosine provide important indirect evidence that peroxynitrite is involved in a pathologic process. Relevant to the participation of peroxynitrite in the MPTP model, it has been demonstrated that MPTP significantly increases striatal levels of nitrotyrosine in mice (75,85). Aside from its role as a marker, nitrotyrosine can be a harmful modification, as it can inactivate enzymes and receptors that depend on tyrosine residues for their activity (86,87) and prevent phosphorylation of tyrosine residues important for signal transduction (88,89). This cascade of events appears quite relevant to MPTP's mode of action, as we have demonstrated that, following MPTP administration to mice, TH becomes inactivated by tyrosine nitration (90). Furthermore, peroxynitrite can damage, through oxidative processes, many vital cellular elements other than proteins (72). Among these, DNA is of unique importance because it is the repository for genetic information and is present in a single copy. Oxidants such as peroxynitrite can cause a

range of DNA damage (72), which can possibly occur in the MPTP model as well as in PD. Indeed, our preliminary data generated in collaboration with Dr. M. F. Chesselet (Department of Neurology, UCLA) indicate that MPTP causes conspicuous DNA damage such as strand breaks in SNc neurons in mice.

CONCLUSION

This summary of a quite prolific decade has attempted to outline the findings and the direction of PD research, which we believe should lay the groundwork for the research that will take place during the coming new millennium. As illustrated above, unquestionable progress has been made toward discovering the etiology and the pathogenesis of the disease. In light of this, and although much work is still before us, we should enter this new era with significant hope and enthusiasm for finding a cure for PD.

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